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Starch pyrodextrins:

In vitro fermentation and physiological effects

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A Thesis submitted for the degree of Doctor in Philosophy

to

The Faculty of Medicine, University of Glasgow (April 2004)

From research conducted at the
Gut, Fermentation and Metabolism Group
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Summary

Starch is a major component of the human diet, which is normally digested in the small intestine and absorbed as glucose. Some starch is resistant to digestion as it is either physically inaccessible (Resistant Starch type 1), of the tertiary structure type B starch, in intact starch granules (Resistant Starch type 2), or retrograded during processing (Resistant Starch type 3). Modified starches such as pyrodextrins may be considered a further type of resistant starch as they are also not digestible and may have some of the physiological actions of resistant starch, such as effects on insulin sensitivity, plasma lipids, promoting the growth of lactic acid bacteria and being fermented in the colon to short-chain fatty acids including butyrate, which is essential to the health of the colonic mucosa and may have several anti cancer effects.

However, the bonds formed in the making of starch pyrodextrins, which are likely to be a mixture of α - and β -bonds may make the structure of the molecules more like a dietary fibre β glucan. The structure of the starch pyrodextrins is not clear. Very little information is available in the published literature; neither are their fermentation and physiological effects well described. In this thesis therefore studies were carried out to evaluate the fermentability of several starch pyrodextrins from Venezuelan crops using a simple *in vitro* model of the human colon, the structure of these pyrodextrins was explored using NMR spectral analysis and their physiological effects were considered in a small pilot study in healthy human volunteers.

The fermentability of lentil, cocoyam and potato pyrodextrins was assessed in an *in vitro* batch culture system, previously used to study the fermentation of resistant starches. Pyrodextrins were produced using an acid catalyst (1.82 g HCl/kg starch, pyroconverting at 140 °C for 3 h) and pre-digested using pancreatic enzymes and amyloglucosidase. Low molecular weight digestive products (< 2000) were dialysed overnight and the larger molecules freeze-dried for use in the fermentation experiments. The pre-digested pyrodextrins were incubated anaerobically with faeces of human subjects in phosphate buffer (pH 6.5) and the residual carbohydrate, gases, culture pH and short-chain fatty acids analysed after 24 h. It was found that pyrodextrinisation changed the short-chain fatty acid profile from 70:13:16 for acetate:propionate:butyrate to 56:27:17 on average. This was true for the three starch sources.

The complete pyrodextrin mixture and the same mixture after dialysis, to remove the low molecular weight (< 2000) components, were analysed by ^1H and ^{13}C NMR spectroscopy. The resulting spectra were compared to published data for known bonds in simple sugars. The results were not conclusive due to complexity of the molecules, but it was highly indicated that there were β bonds, especially the $\beta(1\rightarrow2)$ bond. Much further work is needed to establish these bonds in the pyrodextrins, which is beyond the scope of this thesis. However, there was strong evidence that the anhydro-glucose was separate from the polysaccharides, which is in contrast to current beliefs.

Studies in humans were started to test the effects of these pyrodextrins on faecal short-chain fatty acids and bacteria, and on plasma lipids in mild hyperlipidaemic men. As we could not prepare enough food grade pyrodextrins for these studies,

Canary Islands gofios and Fibersol-2 were evaluated as possible alternatives. These pyrodextrin containing food ingredients were tested for their digestibility and fermentation properties. Gofios had too little resistant carbohydrate to be useful, but had a substantial slowly digestible starch fraction. Fibersol-2 had similar fermentation profile to the tested pyrodextrins. It therefore was selected for the human trial.

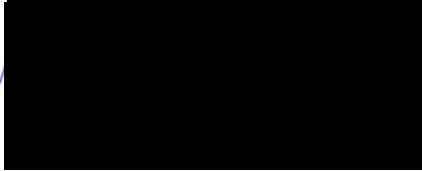
Very slow recruitment however meant that a new simpler pilot study on the effects in normal subjects on faecal short-chain fatty acids and bacteria was carried out instead to test the feasibility of a long-term study. In this study, 11 volunteers ingested 30 g of Fibersol-2 a day or a placebo in yoghurt in a crossover design for one week. Faeces were collected for short-chain fatty acids and bacterial analyses. There was no effect on faecal short-chain fatty acids, but a possible prebiotic effect was demonstrated with an increased growth of Bifidobacteria (about one \log_{10} unit) and Lactobacilli (about half \log_{10} unit).

In conclusion, the pyrodextrinisation of lentil, cocoyam and potato starches changes the structure of the molecule producing low molecular weight species containing anhydro-glucose and larger polymers that are probably highly branched with β glucosidic bonds. These pyrodextrins are not completely digested in the small intestine. However, they are highly fermented giving a higher proportion of propionate yet similar amounts of butyrate to their native starches. This may have implications for plasma lipid levels in humans but sufficient *in vivo* studies were not possible and should be studied in future. The change in the short-chain fatty acid profile may be due to the presence of β bonds in the pyrodextrins or to their solubility, but it was not possible to establish during this

PhD. The *in vivo* actions and potential health benefits of starch pyrodextrins have also to be established. The lipid lowering effects of Fibersol-2 should be confirmed in further studies. It is not clear if these effects will be related to the higher propionate production. Better methods are needed to determine short-chain fatty acid production *in vivo* and particularly the amounts of propionate reaching the liver.

Authors's declaration

I declare that the work contained within this thesis is original and is the work of the author Alexander Laurentin. I have been solely responsible for the organisation and day to day running of this study as well as laboratory analysis and data processing, unless otherwise referenced.



I certify that the work reported in this thesis has been performed by Alexander Laurentin and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow.



Christine A. Edwards

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To the Universidad Central de Venezuela “*La casa que vence las sombras*” (“*The house that overcomes darkness*”) for allowing and encouraging my intellectual development.

Finally, To Dr Christine Edwards, my supervisor and friend, for making First Contact ...and being there!

¡Muchas gracias a todos!

Dedicated to my parents,
María & Guillermo

“Engage!”
Jean-Luc Picard
Captain of the Enterprise

(I’m a Trekkie, I couldn’t help it!)

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List of publications from this thesis

Papers

Laurentin, A. & Edwards, C.A. (2003) A microtitre modification of the anthrone-sulphuric acid colorimetric assay for glucose-based carbohydrates. *Analytical Biochemistry*, **315**, 143-145.

Laurentin, A. & Edwards, C.A. (2004) Differential fermentation of glucose-based carbohydrates *in vitro* by human faecal bacteria: a study of pyrodextrinised starches from different sources. *European Journal of Nutrition* **43**, 183-189.

Articles

Laurentin, A. & Edwards, C.A. Carbohydrates: Resistant starch and oligosaccharides. In *Encyclopedia of Human Nutrition*, 2nd edn, ed. Caballero, B., Allen, L. & Prentice, A. Oxford: Elsevier (*in press*).

Abstracts

Laurentin, A. & Edwards, C.A. (2001) *In vitro* fermentation of pyrodextrinized lentil and cocoyam starches. Presented at the Nutrition Society Diamond Jubilee Summer Meeting, Sheffield, 10-13 July 2001. *Proceedings of the Nutrition Society*, **60**, 192A (Abstract).

Laurentin, A. & Edwards, C.A. (2002) Potato pyrodextrin increases propionate production in *in vitro* culture of human faeces. Presented at 7th International Vahouny Fibre Symposium, Edinburgh, 27-31 May 2002.

Laurentin, A. & Edwards, C.A. (2003) Total and resistant starch content of roasted flours (Canary Islands wheat- and corn-gofio). Presented at the 9th European Conference, Rome, Italy, 1-4 October 2003. *Annals of Nutrition & Metabolism*, **47**, 593 (Abstract).

Chapter 1.

Pyrodextrin, a modified starch

An ordinary day in 1821 was shaken by an event that would change the way British glued their stamps! A textile plant placed on the outskirts of Dublin was on fire. After the fire, a sticky mess was discovered in a room that was used to store starch. This starch which was very damp was identified as just the thing to fill a need in the new postal service. This early record of the production of a starch pyrodextrin resulted in the product called “British gum” which was used for several decades as an adhesive material to glue stamps and envelopes.

Introduction

Pyrodextrin, also known as pyroconverted starch, is a modified starch produced by heat or acid/heat treatment in a low moisture environment. In this chapter, therefore, the starch main features will be considered with special emphasis on their nutritional aspects. Then, a brief introduction on modified starches will be given before a more detailed discussion on pyrodextrins, before a consideration of fermentation by the colonic bacteria and the possible health benefits of the resultant short-chain fatty acids.

1.1. Starch

1.1.1. General aspects

Starch is a carbohydrate used by plants to store energy. It can be found in high amounts in cereals, legumes, roots and tubers. Starchy foods have been very important in human nutrition since pre-historic men settled down in agrarian communities, approximately 10,000 years ago (Richards, 2002). Starch along with

other carbohydrates provide between 40 and 85 % of daily energy in the human diet. Current recommendations suggest that we should obtain at least 47 % of our energy from carbohydrates and at least 37 % should come from starch (Department of Health, 1991). Pyrodextrins which are non-digestible will actually contribute to the fibre component of the diet and, because they are soluble, they may be a way to achieve the 18 g/d recommended (Department of Health, 1991) without influencing other dietary factors.

1.1.2. Structure

Starch is a high molecular weight polymeric carbohydrate composed of D-glucose units. It comprises two different polymers: amylose and amylopectin. Amylose (Figure 1.1) is a lineal polymer in which glucose units are linked together by $\alpha(1\rightarrow4)$ glucosidic bonds, although there are a few $\alpha(1\rightarrow6)$ bonds (Buléon *et al.*, 1998). The molecular weight is variable (500 – 6000 residues) and depends on the botanical source and the extraction method (Buléon *et al.*, 1998). At one end of the molecule, known as the reducing end, there is one glucose residue with one primary hydroxyl group and two secondary hydroxyl groups, in addition to the aldehyde group (as intracatenary hemiacetal). At the other end, known as the non-reducing end, there is a glucose residue with one primary hydroxyl and three secondary hydroxyl groups. The rest of the residues have one primary and two secondary groups (Wurzburg, 1986b).

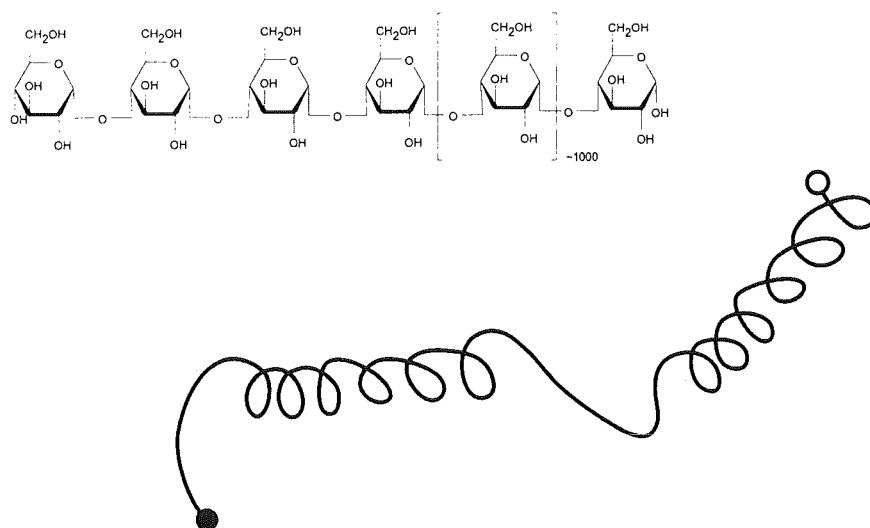


Figure 1.1. Amylose molecule.

Schematic representation of the amylose molecule, showing its chemical structure and helical conformation. The opened and closed circles represent the reducing and the non-reducing ends, respectively.

Amylose chains turn themselves into a helical conformation with six residues per turn (Zobel, 1988). In this conformation, all the hydrophobic groups of the macromolecule face the inner side of the helix and the hydrophilic groups face towards the external side. In this way, an internal hydrophobic tunnel is formed and molecules such as iodine and fatty acids can be bound to it (Gallant *et al.*, 1992).

Amylopectin, on the other hand, is a branched polymer (Figure 1.2). It has mainly glucose units linked by $\alpha(1\rightarrow4)$ glucosidic bonds, but with 5 – 6 % of $\alpha(1\rightarrow6)$ bonds in the branching points (Wurzburg, 1986b; Buléon *et al.*, 1998). These differences in glycosidic bonds and in the secondary structure of the amylopectin alter the way amylopectin is digested in the human small intestine requiring brush

border enzymes to cleave the $\alpha(1\rightarrow6)$ bonds after salivary and pancreatic α -amylase have cleaved the $\alpha(1\rightarrow4)$ bonds. However, the more branched structure makes the bonds more available to enzymes than the tighter helical structure of the amylose molecules and thus amylopectin is more rapidly digested (see below).

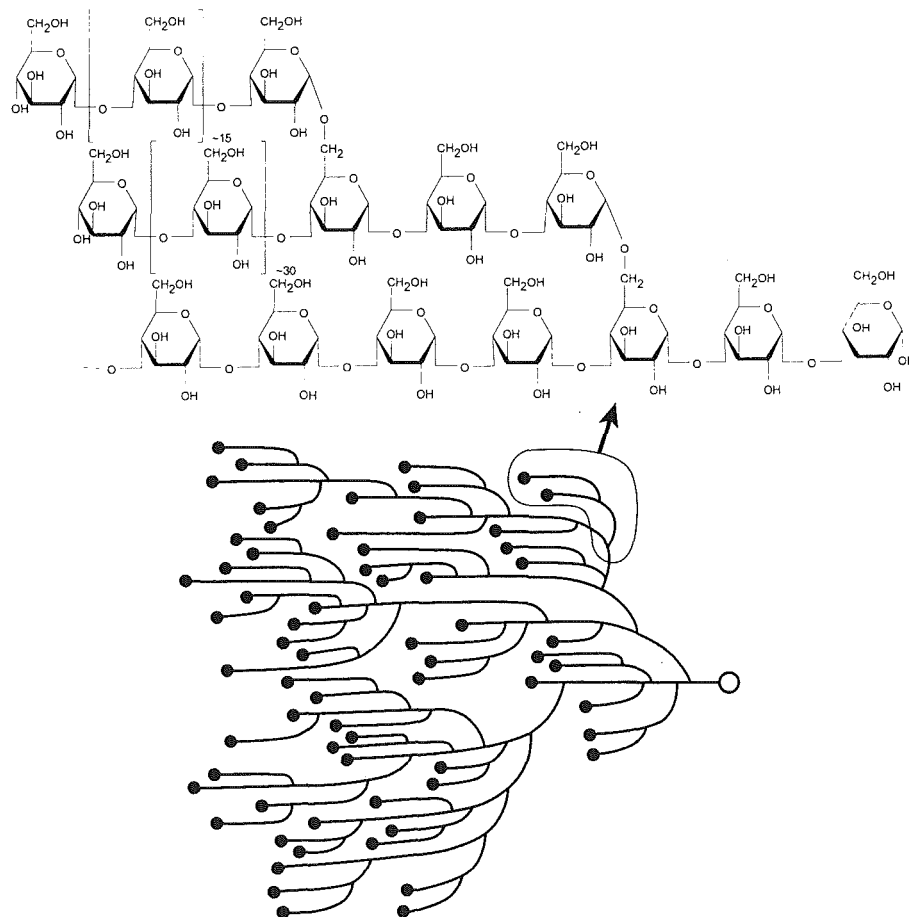


Figure 1.2. Amylopectin molecule.

Schematic representation of the amylopectin molecule, showing its chemical structure and branched clusters. The opened and closed circles represent the reducing and the non-reducing ends, respectively.

1.1.3. Digestion and absorption

Table 1.1 summaries all the steps of starch digestion and fermentation during its passage through the gastrointestinal tract. Food starches can be classified according to the way they are metabolised by the human small intestine in those rapidly digested, those slowly digestible and those resistant to digestion (Englyst *et al.*, 1992).

Table 1.1. Summary of starch digestion, absorption and fermentation processes.

Location	Process
Mouth	Chewing breaks down food, increasing their surface area to volume ratio and exposing starch granules Starch $\alpha(1\rightarrow4)$ bonds begin to be hydrolysed by salivary α -amylase; producing maltose, maltotriose and α -limit dextrins
Stomach	Salivary α -amylase activity is stopped by the acidic environment Further disruption of food by stomach movements and the passage through the pyloric sphincter
Small intestine	Pancreatic α -amylase continues breaking down $\alpha(1\rightarrow4)$ bonds; degrading the starch to the same products as its salivary counterpart Brush border enzymes hydrolyse both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ bonds, releasing glucose from maltose, maltotriose and α -limit dextrins Glucose is absorbed by Na ⁺ -dependent active transporters
Large intestine	Resistant starch (starch that escapes digestion and absorption) is fermented by microflora in the colon producing SCFA and gases (CO ₂ and H ₂) SCFA are absorbed whereas CO ₂ and H ₂ may be converted to methane and acetate
Rectum	Unabsorbed material is voided

SCFA, short-chain fatty acids.

1.1.3.1. Digestible starches

It has become clear that not all starch sources are digested at the same rate.

Englyst has divided starch into rapidly digestible (RDS) and slowly digestible (SDS) starch fractions based on the speed of their digestion *in vitro* (Englyst *et al.*, 1992). RDS are fully digested within 20 minutes in the Englyst assay and SDS within 2 hours. It is difficult to validate these categories *in vivo*, but the concept of slow release or “lente” has long been advocated by David Jenkins’ group as carbohydrates which will produce slow and sustained release of glucose into the blood (Jenkins *et al.*, 1994). Such carbohydrate maybe associated with a lower insulin response and may lead to better insulin sensitivity. This could have major health benefits in terms of diabetes and associated disorders.

1.1.3.2. Resistant starch (RS)

In 1992, a concerted action of European researchers defined resistant starch as “the sum of starch and the products of starch degradation not absorbed in the small intestine of healthy individuals” (Asp, 1992). This concept completely changed our understanding of the action of carbohydrates in the diet because until the early 1980’s, it was thought that starches were completely digested and absorbed in the human small intestine. Three important considerations are attached to this physiological definition. First, resistant starch is made up not only of high molecular weight polymers but also can include dextrins, small oligosaccharides and even glucose, all derived from digested starch that escapes absorption. Secondly, resistant starches reach the human large intestine where they are metabolised by the complex colonic microflora. Finally, the actual amount of resistant starch in a food (i.e. the amount reaching the colon) depends on the physiology of the individual and it may be affected by age.

Resistant starch has also been classified into three types (Englyst *et al.*, 1992): physically inaccessible starch (RS₁), resistant starch granules (RS₂) and retrograded starch (RS₃) (Table 1.2).

Table 1.2. Classification of resistant starch.

Food source	Type	Content in food (g/100g)	Contribution to total RS intake
Cereal products containing whole grains or grain fragments Brown breads Legumes Pastas	RS ₁	1 – 9	Minor
Unripe bananas Uncooked potatoes High amylose starches	RS ₂	17 – 75	Very little
Bread Corn Flakes Cooked cooled potatoes Legumes	RS ₃	1 – 10	Major
Amylose-lipid complex Modified starches	Others	Not known	Unknown

RS₁, physically inaccessible starch; RS₂, resistant granules; RS₃, retrograded starch.
References: Englyst *et al.* (1992) and Englyst & Hudson (2000).

1.1.3.2.1. Physically inaccessible starch (RS₁)

Type I resistant starch is physically inaccessible and is protected from the action of α -amylase, the enzyme that hydrolyses the breakdown of starch in the human small intestine. This inaccessibility is due to the presence of plant cell walls that entrap the starch, for example, in legume seeds (Tovar *et al.*, 1991), partially milled and whole grains. RS₁ can also be found in highly compact processed food like pasta (Björck *et al.*, 1994). The RS₁ content is affected by disruption of the

food structure during processing (e.g. milling) and, to some extent, by chewing (Englyst *et al.*, 1992).

1.1.3.2.2. *Intrinsically highly resistant granule (RS₂)*

Starch granules are plant organelles where starch is produced and stored. Each plant has characteristic starch granules that differ in size, shape, amylose to amylopectin ratio (Hoover, 2001), crystalline to amorphous material ratio, starch supramolecular architecture (Gallant *et al.*, 1992) and amylose-lipid complexes (Tufvesson *et al.*, 2003a), amongst other features. It is believed that combinations of these factors make some granules more resistant to the attack of digestive enzymes than other granules. Type II resistant starch is found in unripe bananas, uncooked potatoes and high amylose starches (Englyst *et al.*, 1992). RS₂ disappears during cooking, especially in water, because a combination of water and heat make the starch gelatinise and therefore giving more access to amylases (Colonna *et al.*, 1992).

1.1.3.2.3. *Retrograded amylose (RS₃)*

Type III resistant starch is the most abundant of the resistant starches present in food. It is formed during usual food processing by cooking and then cooling (Tovar, 1994). When starch is cooked in excess water, it gelatinises, i.e. the granular structure is disrupted, the granule swells and amylose leaks out of the amylopectin matrix. Then when the food is cooled down, amylose (and more slowly amylopectin) recrystallises to a new ordered and more compact structure (a process known as retrogradation) (Colonna *et al.*, 1992), which decreases access for digestive enzymes. RS₃ production can be affected by several factors. For instance, a high amylose to amylopectin ratio (Eerlingen & Delcour, 1995) or a high number of repeated cooking and cooling cycles (Velasco *et al.*, 1997)

increase the amount of RS₃ produced. Retrograded starch can be found in bread, some brands of corn flakes, cooked-cooled potatoes and legumes.

1.1.3.2.4. *Another sources of RS (RS₂)*

In recent years, amylose-lipid complex and modified starches (see Section 1.2.3) have been also recognised as other sources of resistant starches. Amylose-lipid complexes occur when fatty acids (12 – 18 carbons) are held within the helical structure of amylose (Tufvesson *et al.*, 2003b). They are formed naturally during starch biosynthesis, but may also be produced during cooking. Lipids may interfere with amylose retrogradation impairing therefore the production of retrograded starch during processing. However, these complexes themselves have lower digestibility than cooked starch.

In addition to the starch properties already described, several starchy foods (for instance, cereals and legumes) have antinutritional factors, such as lectins, tannins, phytates and enzyme inhibitors (both protease and amylase inhibitors) (Jenkins *et al.*, 1999). Amylase inhibitors present in raw pulses may reduce the activity of amylase in the human small intestine. However, most of these factors, especially enzyme inhibitors, are inactivated during food processing and cooking (Liener, 1994).

1.1.3.2.5. *Physiological effects*

It is very difficult to assess resistant starch intake at present, because there are not enough data on the resistant starch content of foods. In addition, as the resistance of the starch to digestion depends on the method of cooking and the temperature of the food as eaten, the values gained from looking at old dietary intake data may be misleading. Despite this, an average value for resistant starch intake across Europe has been estimated as 4.1 g/d (Asp, 1997). Figures comparable with this

estimation have been made in other countries, for instance, Venezuela (4.3 g/d) (Herrera *et al.*, 2001). It is also very difficult to separate the benefits of slowly, but completely, digestible starches from those that are resistant. In some groups like young children, whose small intestinal digestive capacity is reduced (Christian *et al.*, 2003), the very same food may provide more starch that is resistant for them than for adults.

The main nutritional properties of resistant starch arise from its potential fermentation in the colon. The diverse and numerous colonic microflora ferments unabsorbed carbohydrates to short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate, and gases —hydrogen (H₂), carbon dioxide (CO₂) and methane (CH₄). Acetate is the main SCFA produced (50 – 70 %) and is the only one to reach peripheral circulation in significant amounts, providing energy for muscle and other tissues (Rémésy *et al.*, 1992). Propionate is the second most abundant SCFA and is mainly metabolised by the liver, where its carbons are used to produce glucose (via gluconeogenesis) (Anderson & Bridges, 1986). Finally, butyrate, less abundant than the other two SCFA, is mainly used as fuel by the colonic enterocytes (Roediger, 1980), but also has many potential anticancer properties (Topping & Clifton, 2001) (SCFA are discussed in more detail in Section 1.4.1).

The main physiological effects of digestion and fermentation of resistant starch are summarized in Table 1.3. However, most of the effects shown in Table 1.3 have been observed with a resistant starch intake of around 20 – 30 g/d, which represents 5- to 7- times the estimated intake for the European population (Asp, 1997).

Table 1.3. Physiological effects of resistant starch intake.

Energy	8 – 13 kJ/g; cf 17 kJ/g for digestible starches
Glycaemic and insulinemic response	Depends on food, e.g. legumes (high in RS ₁) and amylose rich starchy foods (which tend to produce RS ₃ on cooking) increase glucose tolerance, but Corn Flakes and cooked potatoes, both with high and similar glycaemic indexes, their resistant starch content are different
Lipid metabolism	Decreases plasma cholesterol and triacylglyceride levels in rat (Lopez <i>et al.</i> , 2001)
Fermentability	Complete, although some RS ₃ are more resistant (Edwards <i>et al.</i> , 1996)
SCFA production	More production than non-starch polysaccharides, especially butyrate (Edwards & Rowland, 1992)
CO ₂ and H ₂ production	Occurs, but to a lesser extent than lactulose (synthetic non-digestible disaccharide in the form Gal β (1→4) Fru)
Faecal pH	Decreased, especially by lactate production
Bile salts	Deoxycholate, a secondary bile salt metabolite with cytotoxic activity, decreases due to the low pH (van Munster <i>et al.</i> , 1994)
Colon cell proliferation	Stimulated in proximal colon, but repressed in distal colon; it seems to be mediated by butyrate
Faecal excretion	Faecal bulk increases due to an increase in bacteria mass and water retention (Phillips <i>et al.</i> , 1995)
Transit time	Do not affect intestinal transit
Nitrogen metabolism	Lower faecal ammonia and phenol excretion (Birkett <i>et al.</i> , 1997)
Minerals	May increase calcium and magnesium absorption in large intestine (Schulz <i>et al.</i> , 1993; Younes <i>et al.</i> , 2001)
Disease prevention	Epidemiological studies suggest prevention against colo-rectal cancer and constipation (Cassidy <i>et al.</i> , 1994; Kritchevsky, 1995)

Fru, fructose; Gal, galactose.

1.1.4. Novel starch sources

In some Latin American countries as Venezuela, starch production and consumption are limited to a few sources, mainly corn and cassava starches. In the year 2000, for instance, the Venezuelan domestic supply of corn and cassava crops accounted for 47 % and 45 % of the cereals and starchy roots, respectively (FAOSTAT data, 2003). However, Venezuela has the potential to produce several non-traditional starchy crops that may increase the option of starch sources. Plants like cocoyam, lentil, Peruvian carrot, rice, sagu, sorghum and unripe plantain are potential starch sources. However, these crops are poorly studied and some of them are produced in small scale, resulting in a considerable number of under exploited crops. In addition, the use of these non-traditional crops may help to develop new starchy products with a variety of functional attributes. Production of these may decrease Venezuelan dependence on imported goods. This point is particular important regarding modified starches, because almost all the modified starches used by the Venezuelan industry are imported. This lack of knowledge, therefore, prompted the studies in the thesis, which consider starches from non-traditional sources, like cocoyam and lentil, along with two major starch sources, like corn and potato.

1.1.4.1. Potato

Potato (*Solanum tuberosum*, Solanaceae) is an annual plant that has underground stolons with edible starchy tubers (Schnee, 1984). The edible portion has 9 % and 80 % (as dry matter) of protein and carbohydrate, respectively, with high water content, 78 % (Instituto Nacional de Nutrición, 1999). The type of X ray diffraction pattern for potato starch is B and the starch granules are large and elliptical in shape (Gallant *et al.*, 1992).

1.1.4.2. Corn

Corn (*Zea mays* L., Poaceae) is an annual gramineous plant that grows between 1 and 3 m height in sub-tropical and tropical regions, its infrutescence has numerous edible caryopses (Schnee, 1984). The edible portion has 11 % and 81 % of protein and carbohydrate (on dry matter basis), respectively, and 14 % water content (Instituto Nacional de Nutrición, 1999). Corn has type A starch (Sahai & Jackson, 1996) and its granules are small and polyhedral (Gallant *et al.*, 1992; Laurentin, 1999).

1.1.4.3. Lentil

Lentil (*Lens esculenta* Medic., Fabaceae) is an annual bush that grows between 30 and 60 cm height in temperate regions. Its fruit is a legume usually with 1 or 2 round flat edible seeds (Schnee, 1984). Lentil seeds have an important nutritional value: 28 % and 65 % (as dry matter) are protein and carbohydrate, respectively, with just 13 % water content (Instituto Nacional de Nutrición, 1999) and relatively low levels of antinutritional factors (Cubero, 1992). Lentil has type C starch (Hoover & Ratnayake, 2002) and its granules have a kidney-like shape (Bhatty, 1995; Laurentin, 1999).

1.1.4.4. Cocoyam

Cocoyam (*Xanthosoma sagittifolium* (L.) Schott., Araceae) is a perennial bush whose roots are starchy edible tubers. It is also known as malanga, tannia and yautia. This plant can grow between 80 and 120 cm in height and its simple leaves are 40 – 90 cm long and 30 – 60 cm width (Schnee, 1984). Cocoyam plants grow in the very wet environment of tropical regions in the Americas, but is not used much as dietary source (Jaffé, 1987). According to the Venezuelan National Institute of Nutrition food composition table, the edible portion of cocoyam roots

has 6 % and 85 % (as dry matter) of protein and carbohydrate, respectively, and high water content, 72 % (Instituto Nacional de Nutrición, 1999). The nutritional value of cocoyam tubers is comparable to potato (Giacometti & León, 1992). Cocoyam has type A starch (Lauzon *et al.*, 1995) and its granules are small and shaped like truncated ellipses (Lauzon *et al.*, 1995; Laurentin, 1999).

1.2. Modified starches

1.2.1. General aspects

There are different types of modified starches that are manufactured by the industrial sector, including the food industry, to improve their functionality and therefore to increase their use. The need of starches that could be gelatinised at higher concentration than their native counterparts, for instance, led to the development of dextrinised starches. The modified starches can be defined as native starches that have been submitted to one or more physical, chemical or enzymatic treatments promoting granular disorganization, polymer degradation, molecular re-arrangements, oxidation or chemical group addition (Wurzburg, 1986b).

1.2.2. Classification

The modified starches can be classified into four main categories, according to their main physicochemical characteristics: pre-gelatinised, derivatised (or substituted), cross-linked and dextrinised starches (Table 1.4). However, they usually are known as physically, chemically or enzymatically modified starch because of the way they are produced (Table 1.5).

Table 1.4. Classification of modified starches.

Starch	Modifying agent	Physicochemical characteristics	Use in food
Pre-gelatinised	Extrusion Drum drying	Soluble in cold water	Cake and instant products
Derivatised	Acetyl Hydroxypropyl Phosphate Diethyl- aminoethyl	Stable at freeze-thawing cycles	Canned and frozen food
Cross-linked	Epichlorhydrine Trimetaphosphate	Stable at higher temperatures, extreme pH, and higher shear forces	Meat sauce thickeners Instant soup Weaning infant food Dressings
Dextrinised	Acid hydrolysis Oxidizing agents (NaOCl) Irradiation (microwave) Heat (pyro-conversion) Amylolytic enzymes	Soluble in cold water Lower or nil viscosity	Chewing gums Jelly Syrups

References: Langan (1986), Pomeranz (1991), Jane (1992) and Zakhia & Chuzel (1993).

Table 1.5. Methods to produce modified starches.

Treatment	Modification	Description
Physically modified	Pre-gelatinisation	Starch paste is pre-cooked and dried by extrusion (González & Pérez, 2002) or drum drying (Supprung & Noomhorm, 2003)
	Dextrinisation	Starch polymers are hydrolysed to smaller molecules by irradiation, e.g. microwaved starch (Tovar <i>et al.</i> , 2001)
Chemically modified	Derivatisation*	Lateral groups are added to starch lateral chains, e.g. hydroxypropylated starch (Kaur <i>et al.</i> , 2004)
	Cross-linking*	Multifunctional groups are used to link two different starch molecules together, e.g. distarch phosphate (Melito, 1998)
	Dextrinisation	Starch polymers are hydrolysed by oxidizing agents (Pereira-Pacheco <i>et al.</i> , 1994), acid hydrolysis (Singh & Ali, 1987), pyrodextrinisation (Laurentin <i>et al.</i> , 2003)
Enzymatically modified	Dextrinisation	Starch polymers are hydrolysed to smaller molecules by incubation with amylases, e.g. syrups (Ande <i>et al.</i> , 1998)

*Double-derived starches are produced by combination of both derivatisation and cross-linking, e.g. hydroxypropyl-distarch phosphate (Kishida *et al.*, 2000)

1.2.3. Digestion and absorption

The digestibility of these modified starches is variable and depends on the kind and extension of the treatment. Some authors have proposed new categories of resistant starch to include not only chemically modified starches (as type IV resistant starch) (Brown *et al.*, 1995), but also physically modified starches and the amylo-lipid complex (Asp, 1997). Indeed, it has been shown that cross-linked starches have 15 – 19 % decreased *in vitro* digestibility when compared with their native starches (Melito, 1998; Tovar *et al.*, 1999b), and hydroxypropylated starch is 50 % digestible (Björck *et al.*, 1989). Moreover, pre-gelatinised starches produced by drum drying and extrusion have a 3 – 6 % and 5 – 11 % decrease in *in vitro* digestibility (Melito, 1998; Tovar *et al.*, 1999a), respectively. Part but not all of this reduction in digestibility is due to the formation of retrograded starch (Tovar *et al.*, 1999a).

In general, addition of chemical groups to the starch polymers may reduce the overall susceptibility to the enzymatic attack of human amylases. On the other hand, physical modifications tend to produce not only retrograded starch, but also unavailable fractions (Tovar *et al.*, 1999a; Tovar *et al.*, 1999b). In both cases, the extent of digestion *in vivo* could be impaired.

1.3. Pyrodextrins

The term dextrin refers, in general, to all starch degradation products no matter how they are produced. In this way, dextrin may refer to enzymatically-hydrolysed starches (like syrups, cyclodextrins) (Ande *et al.*, 1998; Szenté & Szejtli, 2004), physically modified starches (like microwaved starch) (Tovar *et al.*, 2001), acidic catalysed hydrolysis in water suspensions (like acid-modified or converted starches) (Singh & Ali, 2000) and heat-treated starch in dry state (like

pyrodextrins) (Laurentin *et al.*, 2003). This section describes the way pyrodextrins are produced, the chemical changes they undergo and their nutritional significance.

1.3.1. General aspects

Pyrodextrinised starches, pyroconverted starches or just pyrodextrins are starches modified, under low moisture content, by the action of heat or by a combination of heat and an acid catalyst. Pyrodextrinisation is one of the first starch modifying techniques ever reported and was used to produce gum for stamps and envelopes from early 19th century until mid 20th century (Wurzburg, 1986a; Tomasik *et al.*, 1989).

Pyrodextrins have been classified into three categories according to the way they are produced: British gum, white dextrin and yellow (or canary) dextrin. In general terms, white dextrins are produced at low temperatures and acidic pH to minimise development of colour. British gums, on the other hand, are produced at higher temperatures and neutral-to-basic pH; under this condition, a darker colour is developed. Finally, yellow dextrins are more degraded compounds produced by a combination of high temperature and acidic pH (Wurzburg, 1986a; Pomeranz, 1991). This classification is still used even though it is sometimes difficult to clearly define boundaries between the three categories. On the other hand, the use of the term “dextrin” may mislead the reader because it refers to a broader group of molecules. Hence, the use of dextrin by its own will be avoided in this thesis to refer to pyrodextrins.

1.3.2. Pyrodextrinisation steps

From a technological viewpoint, there are four main steps in the production of pyrodextrins: pre-treatment, pre-drying, pyroconversion (or heating) and cooling.

A brief description of each step is provided in the following paragraphs.

1.3.2.1. Pre-treatment

Starch pre-treatment is necessary to achieve the desired pH during the modification process. The preparation of white and yellow dextrins, both requiring low pH, is usually achieved by spraying the starch with a diluted solution of a volatile inorganic acid (like hydrochloric acid) in horizontal or vertical mixers (Evans & Wurzburg, 1967). Alternately, gaseous hydrogen chloride can be also used, especially for the pre-treatment of gelatinised starches, whose granules tend to swell if a solution is used (Wurzburg, 1986a). On the other hand, British gums are prepared with no additive or using salts (like trisodium phosphate, ammonium bicarbonate) to keep the pH neutral or basic. In this case, the mixture can be made by spraying the starch as described above or mixing the salt directly with the starch (Wurzburg, 1986a). A uniform distribution of these additives with the starch is very important (Evans & Wurzburg, 1967) to avoid charring during the heating step.

1.3.2.2. Pre-drying

The pre-drying step decreases the water content that may promote hydrolysis of the starch polymers during heating, especially at acidic pH (Wurzburg, 1986a). This step is, therefore, important for the production of yellow dextrins. It is less critical, however, for the preparation of white dextrins, where hydrolysis is the main reaction involved (see below), and for British gums, where the pyroconversion occurs at higher pH. The pre-drying procedure can be combined

with the pyroconversion step (see below), if the starch is heated slowly and with constant stirring (Wurzburg, 1986a).

1.3.2.3. Pyroconversion

Pyroconversion can be carried out in vertical or horizontal mixers heated directly or by steam or oil jackets. Stirring throughout the process is important to obtain a homogenous product because of an even distribution of heat. Acidity, temperature, rate at which the temperature is raised and incubation time are important variables during the pyroconversion step. White dextrins, for instance, are usually produced between 95 °C and 120 °C, yellow dextrins between 150 °C and 180 °C and British gums between 170 °C and 195 °C (Evans & Wurzburg, 1967). On the other hand, incubation time can range between 30 min (white dextrins) or few hours (yellow dextrins) and up to 18 hours (British gums) (Wurzburg, 1986a). The manipulation of these variables leads to the production of a wide variety of pyrodextrins (Wurzburg, 1986a). In general, an increase in acidity produces pyrodextrins with lower viscosity while higher temperatures makes pyrodextrins with higher degree of branching.

1.3.2.4. Cooling

The pyroconversion step can be stopped when a required property (such as colour, viscosity, solubility or digestibility) is achieved. To do so, the starch can be rapidly cooled down using mixers or conveyor belts equipped with refrigeration systems (usually circulating water). If necessary, the pH can be neutralised at this point by blending the pyrodextrin with alkaline reagents, like ammonium carbonate (Wurzburg, 1986a).

1.3.3. Chemical reactions

The chemical changes that occur during pyrodextrinisation are not totally understood. However, three types of chemical reactions are believed to be involved: hydrolysis, transglucosidation and re-polymerisation. The relative importance of each reaction will depend on the type of pyrodextrin being prepared.

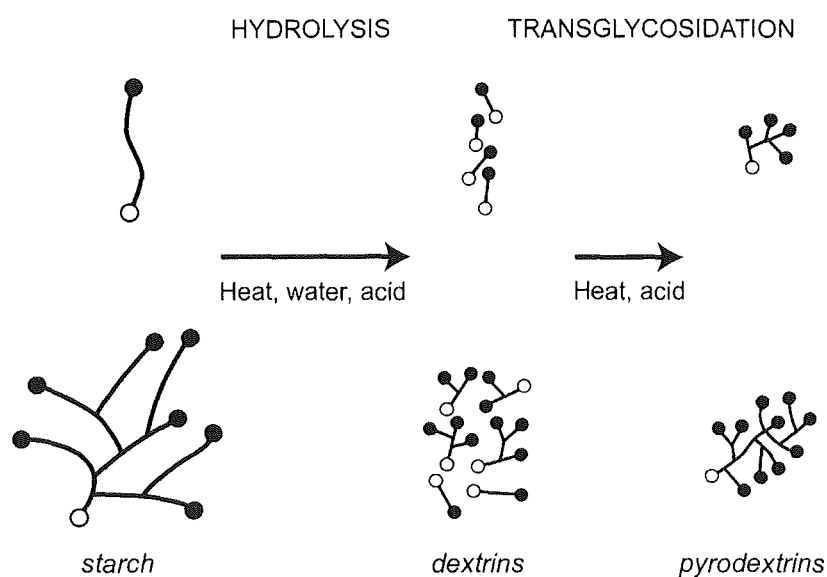


Figure 1.3. Pyrodextrinisation main chemical reactions.

Schematic representation of hydrolysis and transglucosidation reactions during pyrodextrinisation, for amylose (top) and amylopectin (below). The prevalence of each reaction depends on the conditions in which pyrodextrinisation is carried out. The opened and closed circles represent the reducing and the non-reducing ends, respectively.

1.3.3.1. Hydrolysis

This is the first step during pyrodextrinisation and it is characterised by the hydrolysis of $\alpha(1\rightarrow4)$ glucosidic bonds (Figure 1.3) during the pre-drying and the early stages of pyroconversion. Because of the hydrolysis of the starch polymers, the viscosity of the products decreases and the reducing power increases (Wurzburg, 1986a). Hydrolysis seems to be the main reaction that occurs in the white dextrin production.

1.3.3.2. Transglucosidation

The second reaction involved is transglucosidation and it begins to occur when the starch water content is low. This reaction therefore predominates during the manufacture of yellow dextrans and British gums. Briefly, the starch chains break down at $\alpha(1\rightarrow4)$ bonds and react with hydroxyl groups from different chains producing branching points (Figure 1.3). In theory, this chain rearrangement can produce $(1\rightarrow2)$, $(1\rightarrow3)$, $(1\rightarrow4)$ and $(1\rightarrow6)$ glucosidic bonds, in either α - or β -anomers.

However, Theander & Westerlund (1987) reported the presence of maltosan (O- α -glucopyranosyl-($1\rightarrow4$)-1,6-anhydro- β -D-glucopyranose) after enzymatic hydrolysis (with Termamyl and amyloglucosidase) of wheat and potato pyrodextrans (produced by heating at 140 °C for 4 h, with no catalyst added). They postulated that 1,6-anhydro- β -D-glucose (or levoglucosan, Figure 1.4) was present as end units in these pyrodextrans; supporting the pioneer work of Thompson & Wolfrom (1958), who used pyrodextrans prepared by roasting amylose at 185 – 200 °C with no acid catalyst. Moreover, this anhydro-glucose can react further with neighbouring chains to produce branched structures

(Theander & Westerlund, 1987). As the anomeric carbon atom in the levoglucosan has a β configuration, it is likely that the bonds formed have the same anomeric configuration. In fact, some authors (Siljeström *et al.*, 1989) analysing wheat pyrodextrins (180 °C for 4 h, with no catalyst added) using methylation reactions and ^1H -NMR spectroscopy, found the presence of $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ bonds and, in lesser extent, $(1\rightarrow3)$ bond as well.

More recently, some authors studied the pyrolysis of cycloheptaamylose as a model for starch (Lowary & Richards, 1991). Pyrolyses are carried out at more extreme temperatures (usually 250 – 1000 °C) than pyrodextrinisation and at different pressure (for instance, *in vacuo*). Under these conditions, starch produces a variety of volatile and non-volatile compounds (Tomasik *et al.*, 1989). However, low-temperature pyrolysis can provide information about the reactions that take place during high-temperature pyroconversion. Indeed, Lowary & Richards (1991) found that 1,6-anhydro- β -D-glucose was the major product (38 – 50 %) from the vacuum pyrolysis of cycloheptaamylose using different temperatures (280, 300 and 320 °C). They postulated a mechanism of reaction that involves a short-lived glucosyl cation (Figure 1.4): (1) a heterolytic scission of a glucosidic bond produces a seven-member open-chain oligosaccharide with a glucosyl cation at one end, (2) the glucosyl cation is stabilised through an intramolecular attack of O6 on the C1 cation, producing an oligosaccharide with anhydro-glucose at the end, and (3) the anhydro-glucose is released by a subsequent scission of its glucosidic bond, also producing another glucosyl cation.

Lowary & Richards (1991) also proposed that a similar mechanism may operate for pyrodextrins prepared at high temperature. In this case, the heterolytic scission

of the starch chain also produces a glucosyl cation in one end, but the stabilisation of this glucosyl cation may occur by transglucosidation (intermolecular attack) rather than by the intramolecular attack that produces anhydro-glucose. The addition of the glucosyl cation to hydroxyl groups of nearby starch chains results in the branching points. Moreover, it seems that pyrolysis at lower temperatures than usual (171 °C) and atmospheric pressure, (more pyrodextrin-like conditions) favours the transglucosidation reactions over the intramolecular addition (Lowary & Richards, 1990).

1.3.3.3. Re-polymerisation

There is some evidence that a slight increase in viscosity and a decrease in the pyrodextrin reducing power occur during the manufacture of yellow dextrins, especially if long incubation times are used. This has been interpreted as if re-polymerisation of glucose or oligosaccharides into larger molecules is taking place (Wurzburg, 1986a). The high temperature and acidity needed for the production of yellow dextrins may provide a suitable condition for glucosides to polymerise (Ponder & Richards, 1990).

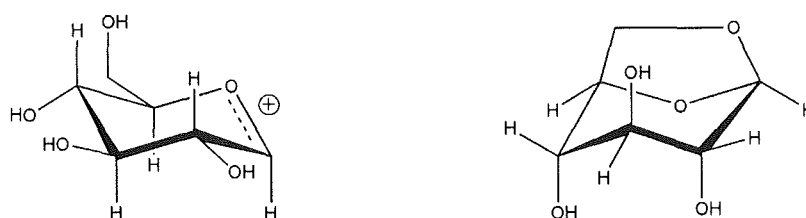


Figure 1.4. Compounds involved in transglucosidation reactions.
Glucosyl cation (left) and 1,6-anhydro-β-D-glucose (right).

1.3.4. Chemical structure

As mention above, pyrodextrinisation is a complicated process where two main chemical reactions (i.e. hydrolysis and transglucosidation) change the chemical structure of the starch, an already complex molecule by itself. Moreover, the different variables involved in the manufacture of pyrodextrins (i.e. pH, water content, temperature and incubation time) make it possible to produce a high number of pyrodextrins with different structures (Wurzburg, 1986a).

These new structures may differ from the known structure of the starch in three aspects: lower molecular weight, higher degree of branching and the presence of non-starch bonds (i.e. not $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow6)$ bonds). More details about the chemical structure of pyrodextrins are presented in Chapter 4.

1.3.5. Functional properties of pyrodextrins

The changes in molecular size, degree of branching, and glucosidic linkages (as mentioned above) define the main functional properties of pyrodextrins. First, a decrease in the molecular size produces a decrease in the viscosity of the modified starch, therefore, pyrodextrins can be prepared at higher aqueous concentrations than their native starch counterpart (Pérez, 1989). Second, an increase in the degree of branching influences the solution stability; making the pyrodextrins soluble in cold water and eliminating the tendency to retrogradation, as reported in the pioneer work of Bernadine Brimhall (1944) using a commercial pyrodextrin prepared in the absent of acid catalyst (British gum). Finally, the formation of non-starch linkages may produce a decrease in the *in vitro* digestibility of the pyroconverted starches (Siljeström *et al.*, 1989; Laurentin *et al.*, 2003).

Consequently, pyrodextrins are soluble in cold water, develop low or nil viscosity in solution and are partially resistant to digestion.

The chemical changes that pyrodextrins undergo also made them a free flowing powder, coloured (from white or light brown to dark brown), with low moisture content (usually 1 – 4 %), no apparent change in the granule morphology and relatively low reducing power (although higher when compared with native starch) (Wurzburg, 1986a; Laurentin, 1999; Laurentin *et al.*, 2003).

1.4. Colonic fermentation

As most of the effect of pyrodextrins are likely to be the results of their fermentation and the SCFA produced, in the next section the process of colonic fermentation and the actions of the SCFA will be discussed. Carbohydrates that enter the colon are fermented by the colonic microflora to SCFA (mainly acetate, propionate and butyrate) and gases (CO₂, H₂ and CH₄). Rapid fermentation produces lactate as an intermediate electron sink (Figure 1.5). Most of the fermentation occurs in the proximal colon although some slowly fermented carbohydrates may move fermentation into more distal regions of the colon. Fermentation results in the loss of chemical structure, water holding capacity reducing impact on stool output and releases any bound or trapped material such as mineral and bile acids. The low pH associated with rapid fermentation has several effects on its own; it precipitates bile and fatty acids, inhibits enzymes such as 7- α -dehydroxylase and inhibits colonic cell proliferation. Starch fermentation has been of particular interest as it tends to increase butyrate production (see below). The fermentation of starch pyrodextrins has not been well studied.

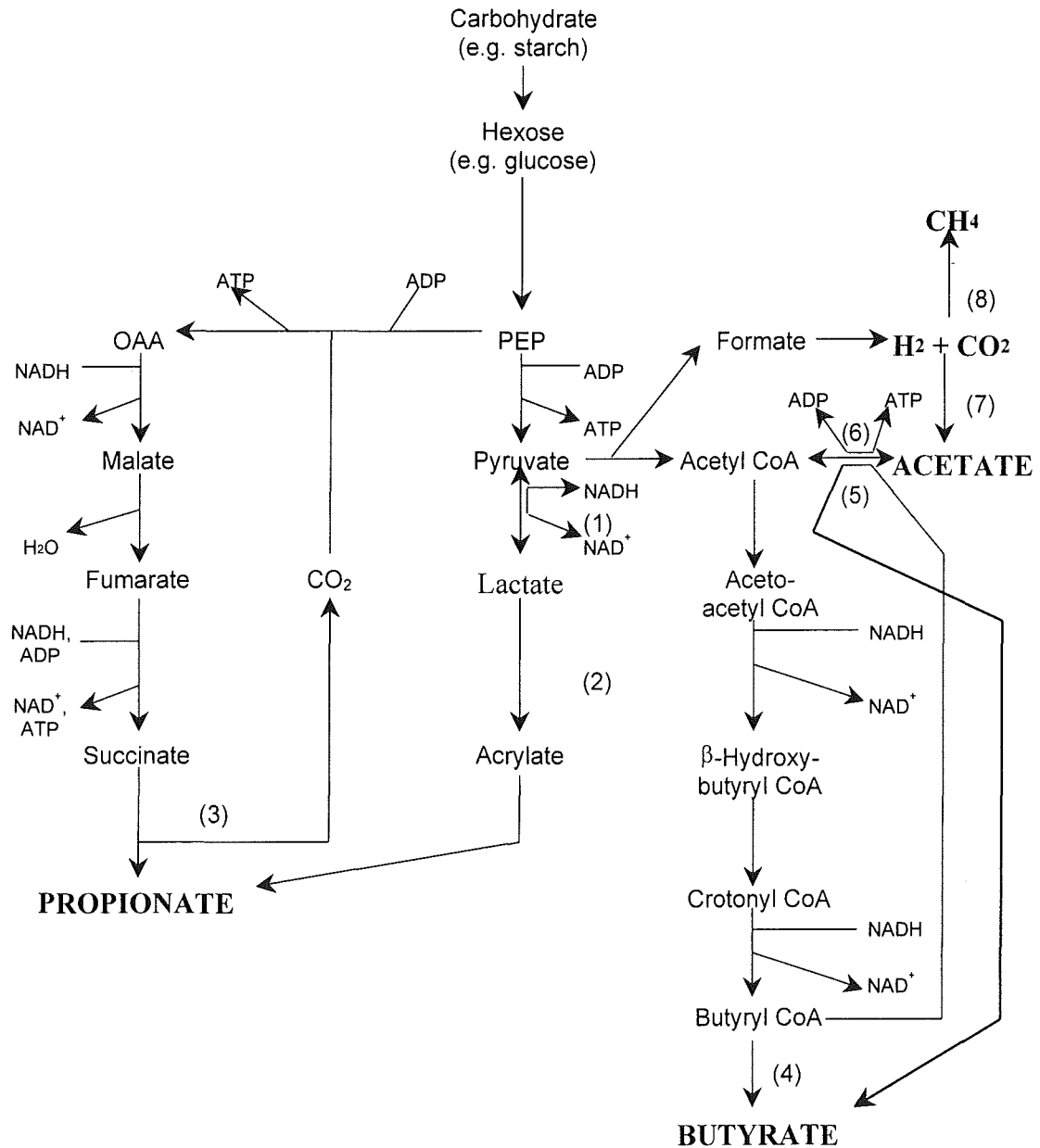


Figure 1.5. Carbohydrate fermentation in the large intestine.

Schematic representation of the main routes of carbohydrate fermentation in the large intestine. OAA, oxaloacetate; PEP, phosphoenolpyruvate; (1), lactate dehydrogenase; (2), acrylate pathway; (3), succinate decarboxylation; (4), phosphotransbutyrylase/butyrate kinase; (5), butyryl CoA:acetate CoA transferase; (6), phosphotransacetylase/acetate kinase; (7), reductive acetogenesis; (8), methanogenesis. Adapted from Pryde *et al.* (2002).

1.4.1. Short-chain fatty acids

SCFA are among the most important products of the colonic fermentation of starches. Acetate, propionate and butyrate are the main SCFA produced (Figure 1.5). However, smaller amounts of valerate and hexanoate (or caproate) are also produced, along with iso-butyrate, iso-valerate (3-methyl-butyrate) and 2-methyl-butyrate. The latter have branched chain structures and are produced by the metabolism of the branched amino acids: valine, leucine and isoleucine (Macfarlane & Allison, 1986).

SCFA produced in the lumen of the large bowel are absorbed by the colonic mucosa by concentration dependent passive diffusion (Vogt & Wolever, 2003) linked to sodium-hydrogen exchange or diffusion through paracellular route or by SCFA-bicarbonate exchange (Velázquez *et al.*, 1996). The venous circulation (portal vein) carries them to the liver before going to the peripheral tissues. Acetate is the only SCFA to reach the peripheral circulation in significant amounts (Pomare *et al.*, 1985), as propionate is used by the liver and butyrate is extensively used by the intestinal mucosa. Recent studies with pigs have shown that the concentration of butyrate in the peripheral circulation is influenced by colonic butyrate production, but this has not been shown in humans, perhaps because pigs eat more fermentable carbohydrate than humans (Bach Knudsen *et al.*, 2003). Propionate has been detected in the peripheral circulation of humans (Wolever *et al.*, 1996) but at much lower level than acetate.

All SCFA seem to have trophic effects on the intestinal mucosa stimulating cell turnover and helping in the healing of damaged intestinal mucosa (Sakata, 1987).

1.4.1.1. Acetate

Acetate is the main SCFA produced (50 – 70 %) from the fermentation of all carbohydrates (Edwards & Rowland, 1992). Acetate is not completely metabolised in the liver and, therefore, is the only SCFA to reach peripheral circulation in significant amounts, providing energy for muscle and other tissues (Cummings *et al.*, 1987). Acetate is also a major substrate for synthesis of fatty acids in the liver (Wolever *et al.*, 1995). In studies where radiolabelled carbohydrates were fed to rats much of the label was found in adipose tissue and 62 % of the label incorporated into the liver was present in the lipid soluble fraction, mainly as phospholipids (Buchanan *et al.*, 1995).

1.4.1.2. Propionate

Propionate is usually the second most abundant SCFA. Bacteroides, propionibacteria and veillonella are the main bacterial groups involved in the production of propionate in the colon. Propionate is mainly metabolised by the liver, where the carboxylation of propionyl CoA produces methylmalonyl CoA, which is rearranged to succinyl CoA, an intermediate of the citric acid cycle. Therefore, the carbons from propionate are used to produce glucose, via gluconeogenesis (Anderson & Bridges, 1986). Propionate has been associated with cholesterol lowering effects. Studies have shown an inhibitory effect of propionate on triglycerides and cholesterol syntheses in the liver that could explain the lipid lowering effect associated with soluble dietary fibres (Chen *et al.*, 1984).

Twenty years ago, Chen *et al.* (1984) suggested that propionate may mediate the cholesterol lowering effect reported for some soluble dietary fibres. When they fed rats with a diet supplemented with 0.5 % sodium propionate plus 0.3 %

cholesterol, both serum and liver cholesterol levels decreased around 15 % compared with rats fed with cholesterol supplemented diet. Although the triacylglycerides in serum did not change, they decreased 35 % in liver. This hypothesis assumed that dietary propionate has the same effect as the propionate produced by colonic fermentation (Chen *et al.*, 1984). However, other authors have suggested that not enough propionate reaches the liver to have this effect (Illman *et al.*, 1988).

The rate of *in vitro* lipogenesis has been measured by the incorporation of tritiated water into fatty acids in hepatocytes isolated from rats (Nishina & Freedland, 1990). Incubating the hepatocytes with different substrates with or without the presence of propionate, these authors found that 1 mmol/L propionate inhibited the rate of lipogenesis from endogenous (i.e. no other substrate added) and from added acetate, butyrate and lactate. Using a similar approach, these authors found no effect from propionate on sterol synthesis. They suggested that propionate (or a propionate derivative) might inhibit acetyl-CoA synthetase, decreasing the activation of acetate into acetyl-CoA (Nishina & Freedland, 1990).

Wolever has shown that, during rectal infusion in humans, acetate was incorporated into serum cholesterol and triglycerides, and raised serum lipid concentration and this effect are blocked by propionate (Wolever *et al.*, 1991; Wolever *et al.*, 1995). In a further study (Wolever *et al.*, 1996), this group compared serum acetate-propionate ratio to total cholesterol and LDL cholesterol levels in humans. They found a significant positive relationship between the serum acetate-propionate ratio with total cholesterol and LDL cholesterol in men, but not in women. Serum propionate was negatively related to serum triglycerides

in men and women, but the effect of women was lost after adjusting for age and body mass index. Care must be taken interpreting these data as acetate is not produced just from the colon but is also produced by the liver in the fasted conditions. Propionate has also been shown to increase insulin sensitivity (Venter *et al.*, 1990; Todesco *et al.*, 1991). However, these studies were carried out using propionate in the diet and not from colonic fermentation. It is worth noting that most propionate predominate fibres, such as guar gum and Ispaghula also tend to reduce plasma cholesterol.

1.4.1.3. Butyrate

Butyrate is usually less abundant than the other two SCFA, but is increased by the fermentation of starch (Englyst *et al.*, 1987), oat bran (McBurney & Thompson, 1987) and fructooligosaccharides (Perrin *et al.*, 2001). Butyrate is mainly used as fuel by the colonic enterocytes (Roediger, 1980). Any butyrate that enters the portal circulation is metabolised in liver towards the production of ketone bodies.

Butyrate is also believed to have many potential anticancer properties, stimulates the growth of colonic mucosa and inhibits the growth and induction of differentiation in colonic cancer lines. Butyrate has been shown to produce growth arrest, differentiation (Augeron & Labois, 1984; Gamet *et al.*, 1992) and apoptosis (Hague *et al.*, 1995) in cancer cells grown in cultures.

Butyrate is also anti-inflammatory, inhibiting production of interleukin 12, tissue necrosis factor α and interferon γ , and may stimulate anti-inflammatory cytokines, such as IL-10 (Saemann *et al.*, 2000; Nancey *et al.*, 2002). More recently, the techniques of genomics and proteomics have been applied to the actions of butyrate in preventing colon cancer. Many genes have been identified which are

altered by butyrate in HT29 cancer cells (Della Ragione *et al.*, 2001; Iacomino *et al.*, 2001; Williams *et al.*, 2003). However, the site of butyrate production in the colon is also important as resistant starch healed experimental colitis (dextran sulfate sodium induced) in the rat colon, but fructooligosaccharides (FOS) did not. The caecal butyrate was higher in the resistant starch fed rats than the FOS fed ones, although distal colonic butyrate was higher in the FOS rats that would appear that caecal butyrate was more important (Moreau *et al.*, 2003).

Butyrate has been shown to heal colitis in patients with Crohn's disease or ulcerative colitis but not if it is given by mouth (Breuer *et al.*, 1991; Steinhart *et al.*, 1994).

It is not clear which bacteria in the colon produce the butyrate. However, cultural and molecular studies have shown that most of these bacteria are strict anaerobes, some of which are previously undescribed species related to *Eubacterium*, *Roseburia*, *Faecalibacterium* and *Coproccoccus* (Pryde *et al.*, 2002).

1.4.2. Colon microflora

Until recently it was commonly believed that the human colon contains a diverse and complex microbial population of $10^{11} - 10^{12}$ bacteria per gram of dry faeces, comprising a stable population of over 400 different bacteria species (Moore *et al.*, 1978). The predominant species are bacteroides, eubacteria, bifidobacteria, Lactobacilli and smaller numbers of clostridia, enterobacteria and streptococci. However, new molecular techniques, which measure bacterial DNA or RNA have suggested that there are many unidentified species. Indeed, in some studies it has been suggested that up to 60 % of the bacterial DNA is unaccounted for by

culturable species. Thus, the field of colonic bacteriology is currently being reinvented (Vaughan *et al.*, 2000).

The enormous number of bacterial species and the interdependence between different species make it difficult to identify the contribution of each species to the fermentation process. However, some species from most bacterial groups can ferment starch (Edwards & Rowland, 1992).

1.4.3. Probiotics and prebiotics

It has been increasingly shown that lactic acid bacteria such as Bifidobacteria and Lactobacilli can increase the health of individuals in terms of the immune function (Isolauri, 2001), resistance to gastrointestinal infections (Pathmakanthan *et al.*, 2000) and perhaps to colonic cancer (Mogensen *et al.*, 2000). These lactic acid bacteria, which have been specifically selected for their health benefits and their survival of the upper gut are known as probiotics. Non-digestible carbohydrates, such as fructooligosaccharides (Gibson & Roberfroid, 1995), can stimulate the growth of such bacteria normally in the gut. These are known as prebiotics. The combination of prebiotics and probiotics, which have a synergistic effect are known as synbiotics (Collins & Gibson, 1999). It has been suggested that resistant starch could also act as a prebiotic and as a component of a synbiotic (Topping *et al.*, 2003). It is possible that the starch pyrodextrin produced in this thesis could have prebiotic actions; such effect has been suggested for a depolymerised pyrodextrin produced from corn (Satouchi *et al.*, 1996).

In conclusion, starch pyrodextrins are an interesting group of molecules with unique, but uncertain structure which could be considered a subfraction of resistant starch. They are not digested in the small intestine, but are fermented in

the large intestine. Their effects on SCFA production are not well established but given their highly soluble properties they can be easily incorporated into the diet. This may provide a health benefit to the colonic mucosa and may influence the microflora in a positive way. There is a very little data on the fermentation and physiological effects of these pyrodextrins. Given the potential advantage of increasing the production of such modified starches from Venezuelan crops, which are at present under utilised, it is important to establish more knowledge of the nature and actions of these molecules.

1.5. Aims of thesis

The aims of this thesis are, firstly, to evaluate the fermentability of several starch pyrodextrins using a simple *in vitro* model of the human colon, secondly, to apply NMR techniques to identify the presence of non-starch bonds in pyrodextrinised starches and, finally, to evaluate the effect of a commercial pyrodextrin, Fibersol-2, on faecal propionate and plasma triglyceride levels in healthy adults.

Chapter 2.

Materials and Methods

This chapter covers the general methods used throughout this thesis. It contains a detailed description of the methods used for starch isolation, pyrodextrinisation and fermentation by human faecal bacterial, as well as the assays used to analyse the fermentation products. Details for the NMR spectroscopy and the human trial design as well as the statistical analyses are given in the individual chapters.

2.1. Starch sources

Among all available starch sources, four different starches were chosen. Two of them, lentil and cocoyam starches, were isolated from Venezuelan non-traditional crops and the other two are major sources of starch in both Europe (potato) and America (corn). Corn and cocoyam have type A starch; potato has type B starch, whereas lentil has type C starch (Gallant *et al.*, 1992; Lauzon *et al.*, 1995).

Lentil (*Lens esculenta* Medic.) seeds and cocoyam (*Xanthosoma sagittifolium* (L.) Schott.) roots were purchased from a local market in Caracas (Venezuela).

Commercially isolated potato (*Solanum tuberosum*, Solanaceae) starch was purchased from Lyckeby Stärkelsen (Kristianstad, Sweden) and isolated corn (*Zea mays* L., Poaceae) starch was supplied by Alfonzo Rivas & Co (Caracas, Venezuela).

2.2. Starch isolation

Cocoyam starch was isolated using the facilities of the Institute of Food Science and Technology (Universidad Central de Venezuela, Caracas, Venezuela). The

isolated lentil starch was kindly supplied by Dr Elevina Pérez (from the above-mentioned Institute). Briefly, decorticated lentil grains or peeled and diced cocoyam roots were homogenised in a liquidiser using one volume of water. The suspension was filtered through a 280-mesh cloth several times (usually 7 times), each time adding one volume of water, until no material passed through the cloth. The filtered material (essentially starch) was washed out with an equal volume of water, three or four times by centrifugation (1500 g for 15 min), removing the lipid layer out of the surface in each centrifugation step until none appeared. Before the last washing, the pH was adjusted to neutrality. Then, the starch was dried in an oven at 45 °C for 24 h, sieved out (250 µm pore), and stored at room temperature (Pérez *et al.*, 1993). In addition to these steps, lentil grains were pre-treated to facilitate their peeling. To achieve this, lentil grains were steeped in 62.5 mmol/L NaOH solution for 24 h at room temperature and washed out extensively before isolating the starch (González Parada & Pérez Sira, 1996).

Yield was 24 % and 45 % (d.m.b.) for lentil and cocoyam starches, respectively. The yields were low because the method described by Pérez *et al.* (1993) was developed for the isolation of amaranth starch and no attempts for improving the yield for these other sources was made because the emphasis was on a high starch purity. In this regard, the starch was assessed using a sequential hydrolysis with Termamyl and amyloglucosidase, and estimating the glucose released by an enzymatic-colorimetric method (Holm *et al.*, 1986). The estimated starch content for these preparations was 94.7 % and 96.8 % for lentil and cocoyam, respectively (Laurentin *et al.*, 2003). Protein content was 0.24 % for lentil (González & Pérez, 2002) and 0.56 % for cocoyam (Pérez, 2001) starches. Therefore, the starting materials for the pyrodextrinisation step were mainly starch.

2.3. Starch pyrodextrinisation

A widely used method for pyrodextrinising corn starch was also applied to cocoyam, potato and lentil starches. Isolated starches (22 g) were sprayed with 0.5 mL HCl (final concentration: 1.82 g acid/kg starch), mixed thoroughly and left overnight at room temperature. The next day, they were heated at 140 °C for 3 h, cooled down to room temperature, milled and sieved through a 250 µm pore size mesh (Ghali *et al.*, 1979).

2.4. Fermentation assessment

The inaccessibility of the proximal colon makes the direct assessment of colonic fermentation in humans very difficult (Edwards & Rowland, 1992). Most of the methods developed, therefore, aim to study fermentation indirectly, by measuring its products in faeces, blood or breath. Although direct measurement of fermentation is easier in animal models, the results may not be extrapolated to humans. In general, *in vivo* methods are expensive, difficult to carry out and have ethical considerations. A comparison between several *in vivo* fermentation methods is summarised in Table 2.1. In Chapter 6, the physiological effect of a commercial pyrodextrin was studied measuring SCFA in faeces. More details about this method are given in that chapter.

A more simplistic approach to assess fermentation is by using *in vitro* models. In these models, human faeces are the source of bacteria for the cultures. It has been shown in post mortem examinations that the microflora composition along the large intestine was relatively constant (Moore *et al.*, 1978). Faeces therefore can provide a representative sample of colonic bacteria and in an appropriate environment (buffer, micronutrients and macronutrients) may mimic the human

colon. The *in vitro* models can be carried out as continuous, semi-continuous or static cultures.

The static (or batch) cultures are simple, inexpensive and can provide information about the extent of carbohydrate fermentation and the profile of SCFA produced. Their use has been standardised through two major ring trials. Barry *et al.* (1995) and Edwards *et al.* (1996) reported the inter-laboratory variation of two batch culture methods. The first one uses fresh human faeces as inoculum and a combination of buffer (carbonate and phosphate), micronutrients and urea (Barry *et al.*, 1995). The second one consists in fresh human faeces and phosphate buffer only (Edwards *et al.*, 1996). Despite their simplicity, both methods showed good agreement between laboratories.

Continuous and semi-continuous cultures, on the other hand, tend to mimic better the colonic environment. They allow keeping the microflora in steady state, and the renewal of substrates and dilution of products, either constantly or at intervals (to mimic the arrival of digesta to the large bowel). However, they are more expensive and time-consuming than batch cultures (Edwards & Parrett, 1999).

The lack of an absorptive surface like in the gut, however, is the main limitation of the *in vitro* cultures, particularly in the static ones, where the fermentation products (SCFA and gases) tend to accumulate; changes in the culture pH and the H₂ partial pressure cause product inhibition (Edwards & Rowland, 1992). Moreover, static cultures show an initial lag phase and a limited life (Khan, 2000).

In spite of these limitations, static cultures are the choice for initial evaluation of the fermentability of food or food ingredients (like pyrodextrins), because of their simplicity, cost effectiveness and reproducibility (Edwards & Parrett, 1999). In

this thesis, the simplest (Edwards *et al.*, 1996) of the batch methods mentioned above was selected to study the fermentation properties of the starch pyrodextrins.

2.4.1. Pre-digestion before fermentation

Since the starch pyrodextrins have digestible components, an *in vitro* pre-digestion step was required to simulate the action of the small intestine before the fermentation experiments. Several *in vitro* methods, which try to imitate human small intestinal digestion, have been developed for the quantification of resistant starch. They differ in the sample preparation (e.g. milled, minced or as eaten), sample pre-treatment (by simulating oral and/or stomach digestion), sample treatment (by using different enzymes mixtures), sample post-treatment (by using KOH or dimethyl sulfoxide as resistant starch solubilising agents) and incubation conditions (shaking/stirring, time, temperature, pH).

For instance, two methods that estimate all the resistant starch fractions include chewing by volunteer subjects as sample pre-treatment. In the Muir method (Muir & O'Dea, 1992), the chewed sample is sequentially treated with pepsin and amyloglucosidase-pancreatic amylase mixture to obtain the non-digestible fraction, which is boiled with Termamyl (thermostable α -amylase) and solubilised with dimethyl sulfoxide to yield finally glucose with another amyloglucosidase-pancreatic amylase mixture step. The Akerberg method is similar to the Muir method, but it includes other steps that permit the estimation of available starch and dietary fibre along with resistant starch (Akerberg *et al.*, 1998). As these two methods need the use of volunteers to pre-treat the samples, their use in this thesis was discarded.

The Berry method (Berry, 1986), on the other hand, measures only RS₃ and RS₂ using an exhaustive incubation (16 hours) of milled sample with α -amylase and pullulanase, following by centrifugation to separate the insoluble residue, which contains the resistant starch. This residue is treated with KOH to disperse retrograded and native starches and then hydrolyse them to glucose with amyloglucosidase. Finally, released glucose is quantified by a colorimetric assay (Berry, 1986). The Berry method has been subsequently modified by Faisant *et al.* (1995) and Goñi *et al.* (1996) by eliminating pullulanase from the enzyme mixture and adding a pre-treatment with pepsin to decrease starch-protein interactions.

The Englyst method was developed to assess all nutritionally important starch fractions, such as rapidly digestible and slowly digestible starches, along with the three types of resistant starches initially described (Englyst *et al.*, 1992). In this method, resistant starch fractions are estimated altogether by difference between total and digestible starches. The first part of the assay was chosen to pre-digest the samples required for the *in vitro* fermentation experiments carried out in this thesis. After a pre-treatment with pepsin, the sample is incubated with a mixture of amyloglucosidase, invertase and pancreatic enzymes during two hours with shaking. This should leave only the physiologically resistant starch-carbohydrate fractions that when eaten will reach the large intestine. In addition, the Englyst method allows the assessment of the different starch fractions (rapidly and slowly digestible starches), information that was used in Chapter 5.

The fermentation method selected (Edwards *et al.*, 1996) in this thesis also uses a modification of the Englyst method to measure the residual starch left after fermentation.

Table 2.1. Comparison between different methods to assess colonic fermentation *in vivo*.

Method	Type of assessment	Invasive	Cost-effectiveness	Medical supervision	Advantages	Disadvantages
Intubation	Direct	Very	Very expensive	Yes	Accurate and quantitative Assess fermentation in its environment	May affect the normal physiology (e.g. change transit time)
Acetate in peripheral blood	Through products	Yes	Less expensive	Yes	Do not affect normal colonic physiology	Endogenous acetate synthesis may interfere
H ₂ in breath	Through products	No	Less expensive	No	Simple, easy to do (commonly used)	Not quantitative (H ₂ has several metabolic fates: CH ₄ , H ₂ S and acetate)
¹³ C in breath	Through products	No	Less expensive	No	Simple, easy to do Can be used in infants and children	Not yet validated Mathematical modelling required
SCFA in faeces	Through products	No	Inexpensive	No	Simple, easy to do	May not reflect colonic fermentation due to absorption and metabolism
Animal model (rat, pig)	Direct and through products	Yes	Expensive	No	Allow long term studies with controlled diets (difficult to achieve in humans)	May not be extrapolated to human Difficult to compare results from different laboratories due to protocol variability

References: Intubation (Florent *et al.*, 1985), H₂ in breath (Rumessen, 1992), ¹³C in breath (Christian *et al.*, 2002), acetate in peripheral blood (Rumessen *et al.*, 1992), SCFA in faeces (Scheppach *et al.*, 1988), animal models (Martin *et al.*, 1998; Le Blay *et al.*, 1999).

2.5. Fermentation of non-digestible fraction *in vitro*

In vitro fermentation experiments were used in Chapters 3 and 5. The samples were pre-digested when appropriate to remove the starch digestible fraction. This pre-digestion step was made using an Englyst starch kit (Englyst Carbohydrate Services Ltd, Cambridge, UK) with some protocol modifications as described fully in Chapter 3.

The *in vitro* fermentation was carried out using human faeces as inoculum in the batch culture model developed by Edwards et al. (1996) and summarised in Figure 2.1. A faecal slurry was made using freshly voided faeces (within 45 min from evacuation), donated by healthy adults (34 – 36 years old), homogenised in 0.1 mol/L Na,K-phosphate buffer, pH 6.5 (pre-boiled, cooled and kept in an oxygen free nitrogen (OFN) atmosphere until used) using a liquidiser. Starch samples (100 mg for test cultures or none in case of control cultures) were suspended in 10 mL 160 g/L faecal slurry (previously filtered through a nylon stocking) in McCartney bottles (28 mL capacity). Each bottle was fitted with a holed, screw cap with a rubber lining to allow flushing of the culture with OFN before incubation. The bottles were incubated horizontally in a shaking water bath (50 strokes/min) at 37 °C for 24 h.

After incubation, produced gas was released and measured using a calibrated syringe. A culture aliquot (2 mL) was used to measure pH and frozen at –20 °C for later SCFA analysis. The remaining slurry was boiled for 30 min and frozen to assess residual starch (Edwards *et al.*, 1996) and carbohydrate. Time zero cultures were immediately boiled for 30 min and frozen to estimate total starch and carbohydrate (Figure 2.1).

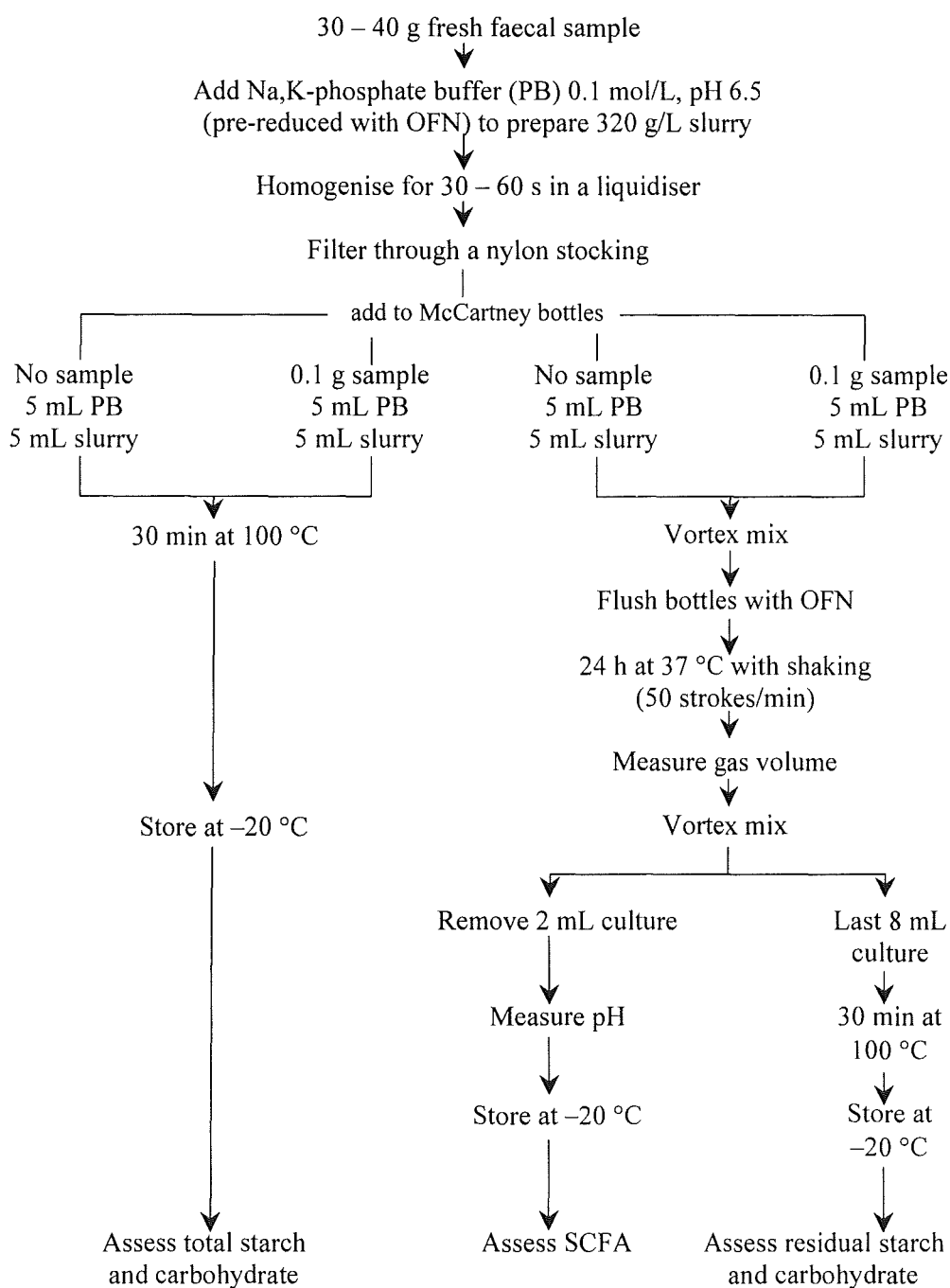


Figure 2.1. Fermentation of non-digestible fraction *in vitro*.

A summary of the *in vitro* fermentation protocol is outline. OFN, oxygen free nitrogen; PB, phosphate buffer.

2.6. Short-chain fatty acid assay

SCFA, the main products of colonic fermentation, were measured in both the faecal *in vitro* cultures (Chapters 3 and 5) and directly from fresh faeces

(Chapter 6). They were estimated by gas liquid chromatography using a TRACE 2000 gas chromatograph (ThermoQuest Ltd, Manchester, UK) equipped with a flame ionisation detector (250 °C) and using a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 µm film thickness), made of polyethylene glycol (catalogue No. 7EK-G007-22, Phenomenex, Cheshire, UK). Nitrogen (30 mL/min) was used as the carrier gas.

Internal standard solution (86.1 mmol/L 3-methyl-n-valeric acid, 0.1 mL) and concentrated orthophosphoric acid (0.1 mL) were added to 0.8 mL culture aliquots (Chapters 3 and 5) or 0.8 mL water containing 50 – 100 mg dried faeces (Chapter 6). The mixture was extracted three times with 3 mL diethyl ether each time, centrifuged and the ether layers pooled. One microlitre of ether extract was automatically injected (230 °C, splitless) into the column. Then, the column temperature was held at 80 °C for 1 min, increased at 15 °C/min until 210 °C and held for 1 min.

The peak integrals were analysed using Chrom-Card 32-bit software version 1.07β5 (2000) by ThermoQuest (Milan, Italy) using an averaged ($n = 5$) molar response factor for each external standard (166.5 mmol/L acetic, 135.0 mmol/L propionic, 113.5 mmol/L isobutyric, 113.5 mmol/L butyric, 97.9 mmol/L isovaleric, 97.9 mmol/L valeric, 86.1 mmol/L hexanoic, 76.8 mmol/L heptanoic and 69.3 mmol/L octanoic acid solutions, pH 8) as calibration method. All the standards were from Sigma-Aldrich Company Ltd. (Dorset, UK), except acetic acid glacial, which was from Fisher Scientific (Loughborough, UK).

Table 2.2 shows representative values for the averaged molar response factors from seven independent assays. The variability of the assay was lower than 2 %

for all SCFA, except for acetate and hexanoate, whose variability was between 2 and 3 %. Additionally, a quality control was included in every run. The quality control used was faecal slurry (160 g/L) prepared as described in section 2.5 and kept in 1.1 mL aliquot vials at -20°C until used. Average values for the quality control ($n = 9$) were 4.53 (SD 0.17), 1.20 (SD 0.07), 1.09 (SD 0.09) and 9.07 (SD 0.35) mmol/L for acetate, propionate, butyrate and total SCFA, respectively.

Table 2.2. Reproducibility of SCFA assay.

SCFA	Molar response factor*	cv [†] (%)
Acetate	0.171 \pm 0.005	2.9
Propionate	0.397 \pm 0.008	1.9
Iso-butyrate	0.608 \pm 0.011	1.8
Butyrate	0.601 \pm 0.011	1.8
Iso-valerate	0.829 \pm 0.015	1.8
Valerate	0.797 \pm 0.014	1.7
Hexanoate	0.985 \pm 0.021	2.1
Heptanoate	1.189 \pm 0.019	1.6
Octanoate	1.372 \pm 0.022	1.6

Values are mean \pm standard deviation ($n = 7$).

* The molar response factor (RF) for each SCFA was calculated using the equation $\text{RF} = (\text{AUC}_{\text{ES}}/\text{AUC}_{\text{IS}})(\text{Conc}_{\text{IS}}/\text{Conc}_{\text{ES}})$, where AUC, area under the curve; Conc, concentration; ES, external standard; IS, internal standard.

[†] Coefficient of variability (calculated as $100 \times \text{standard deviation}/\text{mean}$).

2.7. Starch and Carbohydrate analyses

Starch and carbohydrate were estimated both before (total) and after (residual)

24 h anaerobic incubation to quantify the degree of fermentation (Chapter 3).

Total starch was measured using the enzymatic procedure described by Englyst *et al.* (1992). Residual starch was estimated according to Edwards *et al.* (1996) based upon Englyst *et al.* (1992). These two methods are essentially the same and quantify the starch content as glucose released (see below) after an *in vitro* simulation of both gastric and small intestine enzymatic digestion (protocol details are given in Chapter 3).

The specificity of the Englyst method, however, underestimates the starch content in modified starches such as pyrodextrins because of the presence of non-starch bonds that cannot be hydrolysed by the enzymes used in the method. Hence, measurement of carbohydrate by a more general, chemical assay was necessary to estimate the degree of fermentation of pyrodextrins. The anthrone-sulphuric acid assay was chosen for this purpose. Moreover, the protocol was modified to take advantage of the microtitration plate technology. This matter is described in the next section.

2.7.1. Adaptation of the anthrone-sulphuric acid assay by using microtitration plate technology

Almost 50 years ago, Roe (1955) described a modification of a method to measure dextran in blood and urine (Roe, 1954) for estimation of sugar in blood and spinal fluid. In these methods, carbohydrates are measured using the anthrone-sulphuric acid reaction. Here, heat and a strong acidic environment produce both hydrolysis of glycosidic bonds and dehydration of monomers to produce furfuraldehyde derivatives. These compounds react with anthrone and produce coloured products. However, routine measurement of a large number of samples requires the use of a significant amount of concentrated sulphuric acid and needs strict safety precautions as well as many test tubes. Previous attempts of automation have been hampered by the exothermic nature of this reaction (personal communication from Mr Gustavo Palacios, Universidad Central de Venezuela, Caracas, Venezuela). The use of microtitration plate technology offers a way to overcome these problems and reduce cost, time and hazard. Therefore, I adapted the well-known anthrone-sulphuric acid assay for reliable quantification of glucose-based carbohydrates using 96-well microtitration plates.

This assay requires a total of 12 mL of anthrone-sulphuric acid reagent (2 g/L anthrone solution in concentrated sulphuric acid) and 0.5 mL standard solution (0.4 g/L glucose solution in water) per plate; representing a reduction in the amount of reagent by 8-fold. The anthrone-sulphuric acid reagent must be prepared just before use. Aliquots of 24 mg of anthrone were made in bulk into 30 mL universal plastic containers with leak proof caps and kept protected from light until used. Just before adding the reagent to the plate, 12 mL of concentrated sulphuric acid, stored at 4 °C, were added to one universal container and vortex mixed thoroughly to prepare the reagent.

The microtitration plates employed were 96-well, flat bottomed, polystyrene microtest plates (Immulon 4 catalogue number 011-010-3855, Dynatech Laboratories¹ Ltd., Billingshurst, UK) because they were able to withstand the severe incubation conditions of this assay. In addition, the wells of these plates are joined together at their rim instead of at the bottom. This design allowed each well to be surrounded by water during water bath incubations, promoting a faster and equal distribution of the heat throughout the plate when compared with other plates (Costar catalogue number 9017, Corning Inc., Corning, NY, USA; Ratiolab catalogue number RB/60.1811, Camlab Ltd., Over, UK).

The procedure was as follows: 40 µL water (blank), standard (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.4 g/L glucose) or sample were added to individual wells of the microtitration plate (Figure 2.2). This plate was covered with cling-film, vortex mixed gently and incubated at 4 °C for 15 min. Next, 0.1 mL anthrone solution

¹ Dynatech Laboratories Ltd. has been bought by Thermo Life Sciences Ltd. (Hampshire, UK). According to them, the Immulon microplates 4 HBX (Cat. No. 3855) meet the specifications of the microplates I used from Dynatech.

(freshly prepared) was added to each well using a 12-channel micropipette; the plate was sealed with acetate tape (plate sealer catalogue number M30, Dynatech Laboratories Ltd., Billingshurst, UK), vortex mixed gently but thoroughly and incubated at 92 °C in a non-shaking water bath. After 3 min, the plate was transferred to a non-shaking water bath at room temperature for 5 min to stop the reaction and then placed into an oven at 45 °C for 15 min to dry off. The 3 min incubation is critical as this allows optimal development of colour without deformation of the plate due to the high temperature in the water bath. In this regard, plates should not be left in the water bath for more than 5 min. Absorbance at 630 nm was read in a plate reader (Dynatech MR5000, Dynatech Laboratories Inc., Chantilly, VA, USA) and carbohydrate concentration, as glucose or starch (0.9 x glucose concentration) equivalents was worked out. Under this scheme, 24 samples in parallel with a 7-point standard curve plus blank were assayed by triplicate on each plate. In my hands, the whole procedure was completed within 2.5 hours, which represents 40 % decrease in time, since processing 96 test tubes by the conventional method usually takes 4 hours.

A linear curve was obtained within the concentration range used for the standards (0.05 – 0.4 g/L) represented by the equation: $\hat{Y} = 3.206 X + 0.051$, with 0.982 as coefficient of determination (R^2) and 0.05 as estimated standard deviation (SD) about the regression line. Precision was tested using sample solutions (0.2, 0.3, and 0.4 g/L) of either potato pyrodextrin or commercial glucose-based maltodextrin-like oligosaccharides (Fibersol-2, Matsutani Chemical Industries Co. Ltd., Itami, Japan). In both cases, within run precision was less than 2 % ($n = 3$) and between run precision was less than 4.5 % ($n = 5$). In order to test the capacity of these conditions to hydrolyse a high molecular weight polymer, commercial

soluble starch (catalogue number 33615, Reidel-de Hagaën, Gillingham, UK) was used as standard instead of glucose. The equation obtained ($\hat{Y} = 3.249 X + 0.069$, $R^2 = 0.991$ and $sd = 0.04$) was similar to that of glucose, suggesting that the polymer was completely hydrolysed.

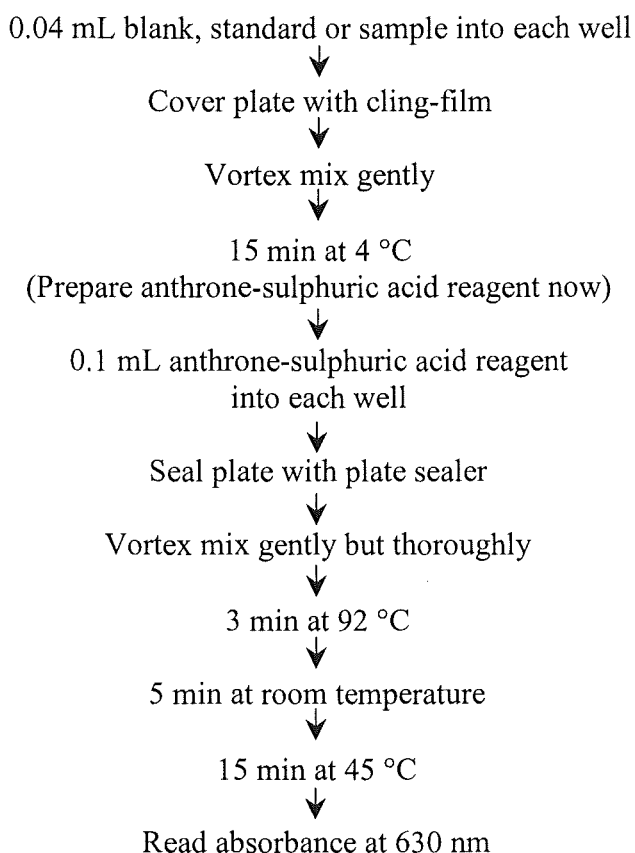


Figure 2.2. Protocol for the carbohydrate assay.

The main steps in the method for analysis of glucose-based carbohydrates by the anthrone-sulphuric acid colorimetric microassay are outlined. Full details are given in the text.

There are other methods to estimate carbohydrate currently in use, especially the method described by Dubois *et al.* (1956), which uses a phenol-sulphuric acid reagent. The Dubois method provides a better way to assay polysaccharides that contain mixtures of different sugar moieties than the assays developed by Roe (Roe, 1954; Roe, 1955). Anthrone reacts differently with different carbohydrate

moieties. As a result it is not accurate to use any one sugar as a suitable standard, when more than one simple sugar is present in the polysaccharide structure. The Dubois method overcomes this limitation by using phenol (Dubois *et al.*, 1956), so a wider spectrum of polysaccharides can be analysed. However, phenol is carcinogenic and may cause adverse mutagenic and teratogenic effects, whereas anthrone in the Roe method has neither carcinogenic nor other known adverse effects. Therefore, when glucose-based carbohydrates like dextrans, starches or modified starches need to be analysed, the anthrone-sulphuric acid assay is the preferred method. It is even more attractive when the assay is performed with small amount of reagents, as the microassay developed, since this reduces the hazard in manipulating corrosive agents like sulphuric acid.

This novel microassay is very suitable for the study of modified starches.

Quantitative analysis of modified starches like pyrodextrans can be underestimated by using highly-specific enzymatic assays (Englyst *et al.*, 1992). This microassay can be used instead, overcoming more complicated and expensive assays like those based on gas liquid chromatography (Quigley *et al.*, 1999). It can also be used as part of the determination of the depolymerisation degree expected from some starch modification procedures by monitoring size exclusion chromatography without using detectors based on diffractometry (Schweizer & Reimann, 1986; Laurentin *et al.*, 2003). This microassay might also be useful in other fields where the anthrone-sulphuric acid assay is being used, such as analysis of carbohydrate mobilisation in mosquitoes (Fadamiro & Heimpel, 2001), quantification of soil carbohydrates (Grandy *et al.*, 2000) and characterisation of organic matter in domestic wastewater (Raunkjaer *et al.*, 1994).

This method was published in *Analytical Biochemistry* (Laurentin & Edwards, 2003) (see Appendix 1).

2.7.2. Glucose assay

The method used to quantify the glucose released from the starch analysis was based on the one described by Bergmeyer & Bernt (1965), which uses the glucose oxidase-peroxidase system. In a first reaction, catalysed by glucose oxidase, D-glucose is transformed into D-gluconic acid with a parallel production of hydrogen peroxide. The second reaction, catalysed by peroxidase, is the oxidation of the chromogen (2,2'-azino-bis-(3-ethyl-2,3-dihydro-1,3-benzthiazol-6-sulfonic acid) diammonium salt or ABTS), which develops a coloured compound.

This assay was also scaled down to use the microtitration plate technology; as a result, some minor modifications were carried out. Briefly, 10 µL water (blank), standard (0.16, 0.20, 0.24, 0.28, 0.32, 0.36 and 0.40 g/L glucose) or sample was added (by triplicate) to individual wells of a microtitration plate. Then, 0.2 mL glucose/GOD-Perid method reagent (Roche Diagnostics GmbH, Mannheim, Germany) was added to each well using a 12-channel micropipette. After 20 min at room temperature, absorbance at 630 nm was read in a plate reader (Dynatech MR5000, Dynatech Laboratories Inc., Chantilly, VA, USA) and starch equivalents (0.9 x glucose concentration) were calculated. A linear curve ($\hat{Y} = 2.511 X + 0.006$, $R^2 = 0.991$ and $sd = 0.03$) was obtained within the concentration range used for the standard.

2.8. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was used as a tool to identify the presence of the non-starch bonds in pyrodextrins. Both ^1H and ^{13}C NMR

spectroscopy were used. The conditions to obtain the spectra and the approaches used to identify the different structural features are given in Chapter 4.

2.9. Culture media

In Chapters 5 and 6, bacteriological analyses were carried out to identify and quantify different bacteria using the following culture media: Beerens, Rogosa and Columbia blood agar for Bifidobacteria, Lactobacilli and general anaerobes, respectively.

Beerens media was prepared as follow: 42.5 g Columbia agar base (CM331, Oxoid Ltd., Basingstoke, UK), 0.5 g cysteine hydrochloride (Sigma, Dorset, UK), 1.5 g agar (for a final concentration of 15 g/L) and 5 mL propionic acid were added to 1 L water. After adjusting pH to 5.0, using 1 mol/L NaOH, the suspension was boiled to dissolve. After boiling, 5.0 g glucose, previously microwaved at full power for 30 seconds (Proline SM11, 750 W, 2.45 GHz), was added per each litre of solution (Beerens, 1991). Once cool enough to handle, the media was distributed into sterile Petri dishes, plates were left to dry at room temperature and then stored at -4°C

Rogosa media (Oxoid CM627) was prepared as labelled by the manufacturer: 82 g were suspended in 1 L water and boiled until the media is completely dissolved. After adding 1.32 mL glacial acetic acid and mixing thoroughly, the solution was heated ($90 - 100^{\circ}\text{C}$) for 2 – 3 min with stirring. The media was added to sterile Petri dishes, dried at room temperature and stored at -4°C .

Plates with Columbia blood agar (CBA; Columbia agar base and defibrinated horse blood) were supplied by E & O Laboratories Ltd. (Falkirk, UK) and stored at -4°C .

On the day of the plating, plates were dried in an oven at 45 – 55 °C for 25 – 35 min before use. All plates were incubated anaerobically in anaerobic jars with Anaerocult A sachets (Merck, Darmstadt, Germany). The anaerobic condition was checked using Anaerotest strips (Merck).

2.10. Human study

The effect of Fibersol-2, a commercial pyrodextrin, on faecal SCFA in healthy humans was investigated in this thesis. Full details of the study design and the methods employed are given in Chapter 6.

2.11. Sample size calculation

To estimate the number of subjects needed for both the faecal *in vitro* cultures (Chapter 3) and the human study (Chapter 6), a sample size calculation was done. To simplify the calculation, it was assumed that the variables would be normally distributed and that a test group would be compared with a control group using unpaired t-test.

During a sample size (n) calculation, it is necessary to define the major output from the study (main variable), estimate the standard deviation (σ) of the main variable, define the minimum difference between groups to be considered important (δ), chose the certainty of detecting the difference between groups (study power) and the significant level (α) (Altman, 1991). The general formula linking these variables is given in Equation 2.1.

Equation 2.1. Formula linking the variables to calculate the sample size.

$$\left(\frac{\delta}{\sigma}\right)^2 = f(\alpha, power) \times \left(\frac{1}{n_1} + \frac{1}{n_2}\right)$$

Where:

δ = the minimum significant difference

σ = the standard deviation (assumed to be the same for both groups)

$f(\alpha, power)$ = the study power and significant level multiplication factor

n_1 = number of subjects required in group 1

n_2 = number of subjects required in group 2

As it was also assumed that both control and test groups would have the same number of subjects, Equation 2.1 could be simplified as Equation 2.2. Finally, a study power of 80 % and a significant level of 5 % were chosen to calculate the sample size. The value for the multiplication factor $f(\alpha, power)$, therefore, was 7.85.

Equation 2.2. Simplify formula to calculate sample size.

$$n = 2 \times f(\alpha, power) \times \left(\frac{\sigma}{\delta}\right)^2$$

Same as Equation 2.1, but assuming that $n_1 = n_2 = n$ (both control and test groups are the same size).

2.12. Statistical analysis

Statistical analyses were calculated using the Minitab for Windows software, release 11.2 32 Bit, 1996 (Minitab Inc., State College, PA, USA) unless otherwise stated. Advice was sought from Dr David Young (Statistician, Research and Development, Yorkhill NHS Trust, Glasgow). Normality of data was evaluated both graphically and using the Anderson-Darling test of normality. The several

statistical analyses used in this thesis are mention in the relevant chapters. A probability level (p) less than 0.05 was used to indicate a significant difference.

Calibration curves (see section 2.7) were obtained using simple regression analysis. In this case, the coefficient of determination (R^2) and the estimated standard deviation (SD) about the regression line are shown.

Chapter 3.

In vitro fermentation of pyrodextrins by human faecal bacteria

Previous studies of *in vitro* digestibility of starch pyrodextrins, carried out before my PhD studies, suggested that a significant amount of the starch was no longer available for enzymatic hydrolysis after pyrodextrinisation. We therefore were interested in the characterisation of the fermentation properties of these pyroconverted starches using a simple *in vitro* model of the human colon. This is the subject of this chapter.

3.1. Introduction

Starch modification techniques have been developed for industrial processing to produce a wide range of potential food ingredients, including pyrodextrinised starches. However, interest in modified starches has been restricted mainly to technological aspects with little concern about the possible impact of the modification on the digestibility and fermentability of the product (Tovar *et al.*, 1999a; Tovar *et al.*, 1999b).

As mentioned in Chapter 1, pyrodextrins are produced by reactions that take place under the influence of heat, often in the presence of catalytic amounts of ions. These modified starches were first reported nearly 200 years ago, as a water soluble and gummy material from the roasting of starch (Tomasik *et al.*, 1989). Native starch is composed of glucose units linked by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glucosidic bonds, which can be broken down by the digestive enzymes (α -amylase and α -dextrinase) present in the small intestine. Starch pyrodextrinisation occurs because of hydrolysis, transglucosidation and, in some

cases, re-polymerisation reactions of glucans. The significance of each reaction depends upon the conditions in which pyrodextrins are prepared (Wurzburg, 1986a). Transglucosidation reactions, for instance, predominate in the formation of yellow dextrins (i.e. pyrodextrins prepared in an acidic environment and at high temperature, like in this thesis). Glucosyl cation, produced by the heterolytic scission of the glucosidic bond, has been postulated as intermediate (Lowary & Richards, 1991). It can undergo either intramolecular attack (producing 1,6-anhydro- β -D-glucose in one chain end) or intermolecular attack of neighbouring chains (producing branching points). The latter seems to predominate during the production of pyrodextrins (Lowary & Richards, 1990; Lowary & Richards, 1991). Therefore, pyroconverted starches are branched, low molecular weight products showing new glucosidic linkages. It is claimed in the European patent EP 0 540 421 A1 (Ohkuma *et al.*, 1993a) that the non-digestible fraction of a potato pyrodextrin had one-third of its glucose residues at the non-reducing end of a chain, and one-sixth of glucose residues presented non-starch linkages, such as (1 \rightarrow 3) and (1 \rightarrow 2). In addition, four main groups of molecular size for pyrodextrinised corn starch with apparent MW at 5000; 10,000; 19,000 and 40,000 have been described (Laurentin *et al.*, 2003).

Although it has been recognised for some time that pyrodextrins prepared without any catalyst were resistant to digestion (Siljeström *et al.*, 1989), data about the digestibility and fermentability of pyrodextrins (with or without catalyst) are not readily available. Recently, other authors have found not only that acid catalysed pyroconversion promoted the generation of a significant proportion of non-digestible fractions (around 60 %), but also these changes differed in magnitude depending on the starch source (Tovar *et al.*, 1999b; Laurentin *et al.*,

2003). A decrease in rat caecal content pH and an increase in butyrate was reported when rats were fed a potato or corn pyrodextrin enriched diet in comparison with a corn fibre diet (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b; Kishimoto *et al.*, 1995). However, full characterisation of the fermentation of pyrodextrins from other plant sources and in comparison to their native starches has not been reported.

In recent years, resistant starches have been recognised for the contribution they can make to human health throughout their interaction with the gut. In particular, the increased butyrate production, as this short-chain fatty acid (SCFA) may have anticancer and anti-inflammatory effects on the colo-rectal mucosa (Scheppach *et al.*, 1992; Bird *et al.*, 2000). Pyrodextrinised starches share some of the properties of resistant starch. They are partially fermented, producing more butyrate than non-starch polysaccharides, gases (CO₂ and H₂) and a low colonic pH (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b; Kishimoto *et al.*, 1995). On the other hand, pyrodextrinised starch, but not resistant starch (Asp, 1997), decreased gut transit time in rats (Wakabayashi *et al.*, 1992a) and may lower serum cholesterol and neutral fat in humans (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b; Ohkuma & Wakabayashi, 2001). As pyrodextrinised starches have different glucosidic bonds from resistant starch, they may be fermented by different bacterial species and both the process and end result of fermentation may differ.

The aim of this work, therefore, was to evaluate the effect of pyrodextrinisation on the fermentation characteristics of starches, prepared from potato, lentil and cocoyam, as a substrate in a simple *in vitro* model of the human colon.

3.2. Materials and Methods

3.2.1. Starch sources

Lentil, cocoyam and potato starches, both native and modified, were used for this study. Their source, isolation procedure and pyroconversion were described in Chapter 2.

3.2.2. Starch pre-digestion

To remove the digestible starch both native and pyrodextrinised starches were pre-digested using an Englyst starch kit (Englyst Carbohydrate Services Ltd, Cambridge, UK) with some protocol modifications, as outlined in Figure 3.1. Briefly, 0.8 g starch and 50 mg guar gum were weighed in a centrifuge tube, then 10 mL of 5 g/L pepsin (Sigma-Aldrich Company Ltd., Dorset, UK) suspension in 50 mmol/L HCl were added, vortex mixed and incubated for 30 min at 37 °C. To simulate small intestinal digestion, five glass balls and 10 mL 0.25 mol/L sodium acetate solution were added to each tube and they were shaken well by hand. After equilibration at 37 °C, an enzyme mixture (pancreatin from porcine pancreas, Sigma-Aldrich; amyloglucosidase from *Aspergillus niger*, Novo Nordisk, Bergen, Norway and invertase from yeast, Merck, Leicestershire, UK; 5 mL) was added and incubated for 2 h in a shaking water bath (70 strokes/min) (Englyst *et al.*, 1992).

Then, the glass balls were taken out and the tubes were spun at 1500 g for 5 min. The non-digestible fraction from the pre-digested native starches was recovered from the pellets, freeze-dried, pooled, milled in a mortar and used for the fermentation experiments. This fraction contained the resistant starch, as defined by Englyst *et al.* (1992), and guar gum.

In contrast, the non-digestible fraction from the pre-digested pyrodextrinised starches remained in the supernatants after centrifugation, due to their water-solubility. However, as the products of the pre-digestion step were also present in the supernatant, these were removed by dialysis (overnight dialysis against water at 4 °C, followed by five 1-hour repeat dialysis steps with stirring) using dialysis bags with low MW cut-off (2000; Sigma-Aldrich). Retentates were pooled, freeze-dried, milled in a mortar and used for fermentation. This fraction contained the non-digestible component from the pre-digested pyrodextrins and the enzymes from the starch kit (Figure 3.1).

As the SCFA profile was one of the variables studied, it was important to consider the effect of other fermentable substrates present in the system. Therefore, potato native and pyroconverted starches were fermented as described in Chapter 2, but without the pre-digestion step to look at the impact of the presence of either guar gum or the enzymes (from the starch kit) in the non-digestible fractions obtained from native or pyrodextrinised starches, respectively. There were no discernible effects of either component.

3.2.3. Fermentation of non-digestible fractions

Non-digestible fractions (and glucose, as a readily fermented control) were fermented according to Edwards *et al.* (1996) in four to six independent determinations (actual sample size is given in Table 3.1). SCFA, pH and volume of gas produced were measured as fermentation products. Full details of these procedures are given in Chapter 2.

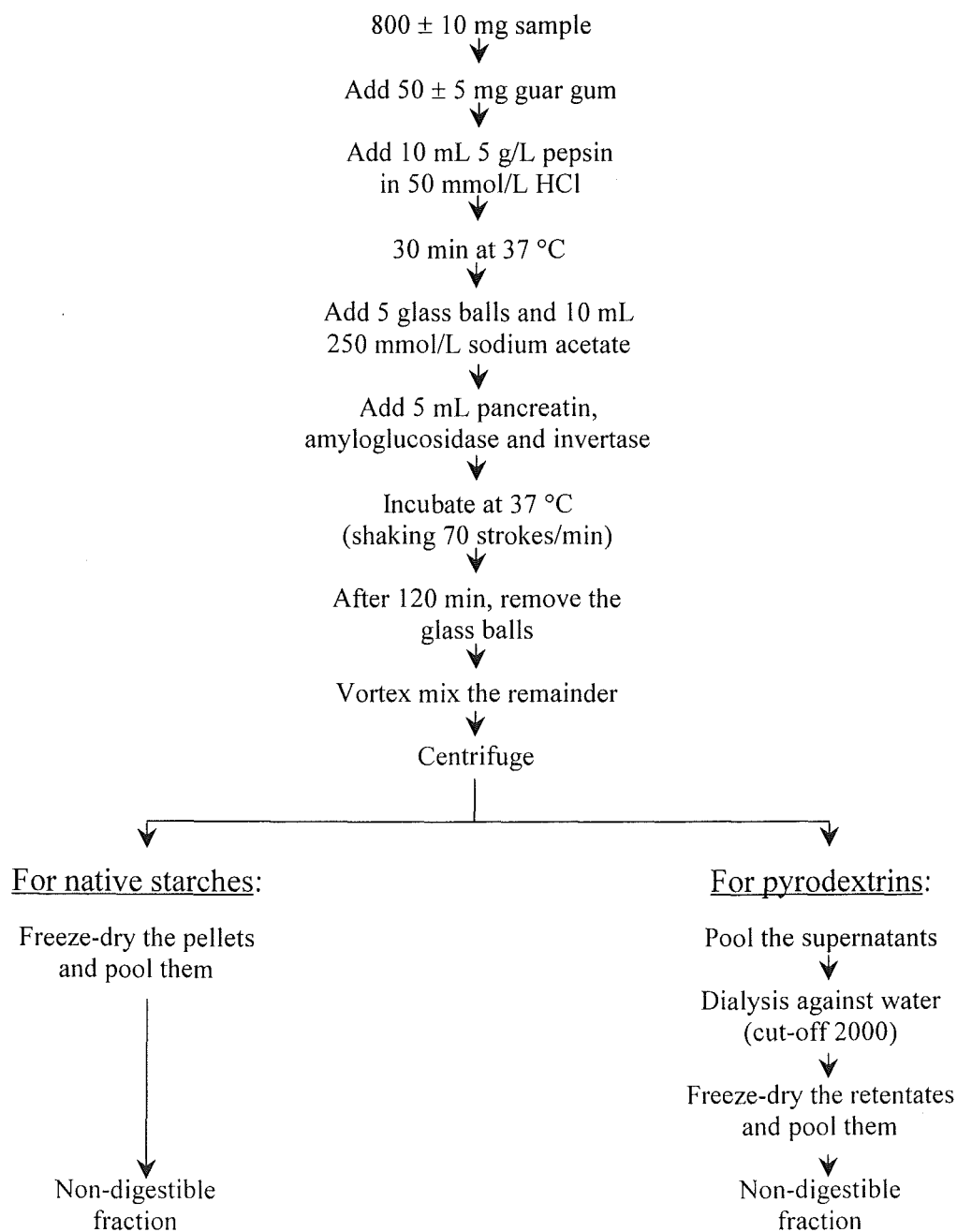


Figure 3.1. Pre-digestion protocol.

A summary of the protocol for sample pre-digestion, based on Englyst *et al.* (1992) is outlined. More details are given in the text.

The sample size for the fermentation experiments was calculated using the equation and the assumptions described in Chapter 2. From preliminary *in vitro* fermentations of native lentil starch, the propionate proportion was chosen as the main variable of this experiment and estimated as 10.7 (SD 1.6) %, $n = 3$. An increase of at least 30 % of this value will be considered as a significant difference ($\delta = 3.2$), therefore, a sample size of four subjects for native and pyrodextrinised starch groups would be needed.

3.2.4. Starch and Carbohydrate analyses

Starch and carbohydrate were estimated both before (total) and after (residual) 24 h anaerobic incubation to quantify the degree of fermentation. Total starch was measured using the enzymatic procedure described by Englyst *et al.* (1992). Residual starch was estimated according to Edwards *et al.* (1996) based upon Englyst *et al.* (1992). These two methods, outlined in Figure 3.2, are essentially the same and quantify the starch content as glucose released after an *in vitro* simulation of both gastric and small intestine enzymatic digestion. The released glucose was quantified using the glucose oxidase-peroxidase system, scaled down to use microtitration plates, as described in Chapter 2.

The specificity of the Englyst method, however, underestimates the carbohydrate content in modified starches such as pyrodextrins because of the presence of non-starch bonds (i.e. not $\alpha(1 \rightarrow 4)$ or $\alpha(1 \rightarrow 6)$ bonds) that cannot be hydrolysed by the enzymes used in the method. Therefore, measurement of carbohydrate by a more general, chemical assay was necessary to estimate the degree of fermentation of the pre-digested pyrodextrins. Total and residual carbohydrates were estimated, by sampling from the same preparations made for total and

residual starch analyses (Figure 3.2), using the anthrone-sulphuric acid method, modified to perform the assay into microtitration plates, as described in Chapter 2.

Starch and carbohydrate analyses were carried out for both native and modified starches. Appropriate calibration curves were made with each plate using glucose as standard. The standard solutions were prepared dissolving 10 μ L of 40 g/L glucose standard solution in 1 mL slurry supernatants (1500 g for 5 min) from control cultures at time zero to correct for the glucose present in the amyloglucosidase suspension and any possible interference from the slurry supernatants.

3.2.5. Statistical analysis

Variables were described as mean \pm standard deviation. One- or two-sided unpaired t-test was used to compare means of pre-digested pyrodextrinised against pre-digested native starches where appropriate.

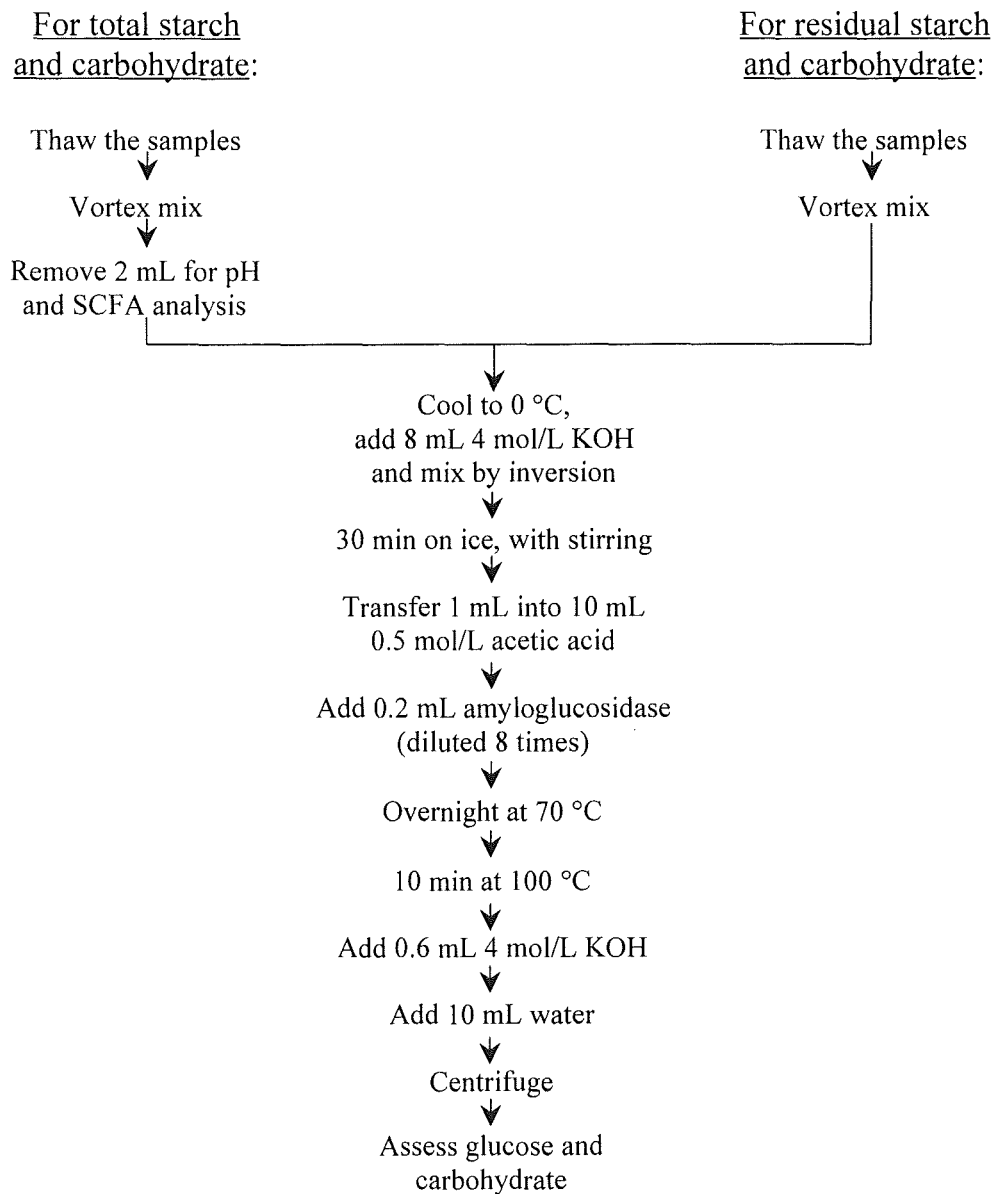


Figure 3.2. Total and residual starch and carbohydrate protocols.

A summary of the protocols, based on Edwards et al. (1996) and Englyst et al. (1992), to measure both starch and carbohydrate, before (total) and after (residual) fermentation is outlined. Glucose and carbohydrate assays are described in Chapter 2.

3.3. Results

3.3.1. Fermentation of native and pyrodextrinised starches

The fermentation properties of both pre-digested native and pyrodextrinised starches from several sources after 24 h *in vitro* anaerobic incubation with human faeces are shown in Table 3.1 and Table 3.2. In cultures containing the pre-digested native starches, 99 %, 98 % and 95 % of potato, lentil and cocoyam starches, respectively, were fermented (Table 3.2). Although a similar trend was found for the pre-digested pyrodextrinised samples, the modified starch was measured as carbohydrate content by a chemical method to overcome the specificity of the enzymatic starch assay (Englyst *et al.*, 1992). With this approach, 77 %, 75 % and 81 % of the carbohydrate in potato, lentil and cocoyam pre-digested pyrodextrins were fermented, respectively (Table 3.2).

3.3.2. Total SCFA

The net total SCFA, estimated as mmol/L of fermented culture, was similar for all starches (Table 3.1). Comparable amounts were produced using glucose as substrate (58.8 (SD 4.4) mmol/L, n = 6). However, when net total SCFA were corrected for initial culture carbohydrate content (Table 3.2), SCFA production remained similar for all the pre-digested native starch sources, but the total SCFA for the pre-digested pyrodextrinised starches were significantly higher (between 43 % to 75 %) than their corresponding pre-digested native starches ($p < 0.023$, one-sided t test) (Table 3.1). Control cultures (n = 6), i.e. those fermented without starch samples, produced 32.4 (SD 9.1) mmol/L of total SCFA, achieved pH 6.5 (SD 0.1) and produced 7 (SD 3) mL gas.

3.3.3. Molar proportions of SCFA

All pre-digested pyrodextrins showed a significantly higher ($p < 0.017$, one-sided t test) molar ratio of propionate (around two fold) when fermented compared with their pre-digested native starch. In addition, the acetate molar ratio decreased ($p < 0.04$, one-sided t test) by about 24 % (Table 3.1). There was no difference in the n-butyrate molar proportion ($p > 0.82$). The statistical significance of these changes was not modified when two-sided t-test were used, except for the variation in the acetate proportion for potato.

3.3.4. Fall in pH and gas produced

The culture pH was 6.6 (SD 0.1), $n = 6$ at time zero. After fermentation, all the cultures showed a decrease in pH, but the fall in pH was significantly less for the pre-digested pyroconverted samples ($p < 0.03$, one-sided t test). The fall in pH observed for glucose (1.7 (SD 0.3), $n = 6$) was similar to all the pre-digested native starches (Table 3.1). The volume of gas produced was the same for all samples, including glucose (17 (SD 5 mL), $n = 6$).

Table 3.1. Short-chain fatty acid, fall in pH and gas produced in fermented cultures of pre-digested native and pyrodextrinised starches from potato, lentil and cocoyam[‡].

	Starch source (pre-digested starches)					
	Potato		Lentil		Cocoyam	
	Native (n = 5)	Pyrodextrin (n = 4)	Native (n = 6)	Pyrodextrin (n = 6)	Native (n = 4)	Pyrodextrin (n = 6)
Net total SCFA [§] (mmol/L)	55.4 ± 5.8	61.0 ± 5.8	58.2 ± 5.0	56.6 ± 4.4	60.1 ± 14.5	59.1 ± 5.4
Net total SCFA ^{‡,§} (mol/kg initial CHO)	6.9 ± 0.7	10.4 ± 1.9*	7.0 ± 0.4	10.0 ± 0.7***	6.8 ± 2.3	11.9 ± 1.8*
Net total SCFA (mol/kg fermented CHO)	7.9	13.2	7.6	13.5	10.0	14.6
Individual SCFA proportion (%)						
Acetate [†]	64.9 ± 12.6	51.1 ± 3.5*	68.8 ± 12.1	51.4 ± 3.0**	66.0 ± 7.0	51.3 ± 3.1*
Propionate [†]	14.7 ± 3.4	25.4 ± 5.9*	12.0 ± 3.2	24.3 ± 5.6**	11.1 ± 1.7	24.4 ± 5.2***
n-Butyrate	15.4 ± 8.3	15.1 ± 5.1	14.8 ± 7.9	15.6 ± 4.1	16.1 ± 2.9	16.1 ± 4.1
iC4–C8 [†]	5.0 ± 3.2	8.5 ± 4.1	4.4 ± 3.4	8.7 ± 3.2*	6.9 ± 4.8	8.2 ± 3.7
Fall in pH [†] from 6.6 ± 0.1	1.5 ± 0.3	1.0 ± 0.1**	1.6 ± 0.3	0.9 ± 0.1***	1.4 ± 0.2	1.0 ± 0.1*
Gas produced (mL)	16 ± 3	20 ± 4	14 ± 8	17 ± 3	10 ± 5	15 ± 6

Values are means ± standard deviations.

CHO, carbohydrate; SCFA, short-chain fatty acids; iC4–C8, sum of iso-butyrate, iso-valerate, valerate, hexanoate, heptanoate and octanoate.

Mean values were significantly different from their corresponding pre-digested native starch: *p < 0.05, **p < 0.01 and ***p < 0.001 (unpaired t-test, [†]one-sided). Note: two-sided t-tests also showed significance (p < 0.05) for the same variables, except 'Acetate' for potato and 'Fall in pH' for cocoyam.

[‡] Fermentation was carried out in anaerobic incubations with human faeces for 24 h.

[§] Net total SCFA was calculated by subtracting total SCFA values in control cultures (no carbohydrate) from total SCFA values in test cultures. Total SCFA in faecal slurry (at time zero) was 11.4 ± 4.8 mmol/L (n = 6).

^{||} The amount of fermented carbohydrate was calculated by subtracting 'Residual carbohydrate' values from 'Total carbohydrate' values (Table 3.2).

Table 3.2. Starch and carbohydrate contents in fermented cultures of pre-digested native and pyrodextrinised starches from potato, lentil and cocoyam[†]

	Starch source (pre-digested starches)					
	Potato		Lentil		Cocoyam	
	Native (n = 5)	Pyrodextrin (n = 4)	Native (n = 6)	Pyrodextrin (n = 6)	Native (n = 4)	Pyrodextrin (n = 6)
Total starch ^{†,§} (mg SE)	78.8 ± 2.4	5.6 ± 1.8***	85.7 ± 11.0	7.0 ± 6.5***	90.5 ± 3.2	5.6 ± 3.3***
Residual starch ^{†,} (mg SE)	1.1 ± 0.7	0.8 ± 0.9	1.6 ± 1.1	0.7 ± 0.6	4.3 ± 2.4	0.6 ± 0.6*
Total carbohydrate ^{†,§} (mg SE, n = 4)	77.7 ± 8.1	59.8 ± 8.3*	80.5 ± 2.3	55.7 ± 3.9***	87.3 ± 2.9	50.2 ± 4.3***
Residual carbohydrate (mg SE, n = 4)	7.4 ± 6.0	13.5 ± 4.4	4.1 ± 3.6	13.9 ± 2.2*	27.1 ± 13.3	9.6 ± 2.2

Values are means ± standard deviations.

SE, starch equivalents (0.9 x glucose concentration).

Mean values were significantly different from their corresponding pre-digested native starch: *p < 0.05 and ***p < 0.001 (unpaired t-test, [†]one-sided). Note: two-sided t-tests also showed significance (p < 0.05) for the same variables, except 'Residual starch' for cocoyam.

‡ Fermentation was carried out in anaerobic incubations with human faeces for 24 h.

§ Before fermentation.

|| After fermentation.

3.4. Discussion

Corn, potato and cassava starches account for almost 90 % of the starch produced in the world (Ostertag, 1996). To widen the utilisation of under exploited crops such as lentil and cocoyam, produced in Latin American countries like Venezuela, as starch sources, it is important to study their characteristics as potential food ingredients. Previous work has shown that pyrodextrinised starches produced by the heat-acid treatment used here have a 55–65 % decrease in the enzymatically available starch. Therefore, pyrodextrins are an excellent source of non-digestible carbohydrates (Laurentin *et al.*, 2003). Moreover, these pyrodextrins differ from other short-chain saccharides because they are composed of a complex mixture of starch derivatives, all with MW below 105,000 as estimated by gel filtration chromatography (Laurentin *et al.*, 2003).

However, the water solubility of the pyrodextrins caused an analytical problem. During the pre-digestion stage, the non-digestible fraction from pre-digested native starches is normally obtained as a pellet after centrifugation at the end of pre-digestion (Englyst *et al.*, 1992). In contrast, the pyrodextrinised starches remain in the supernatant along with their products of digestion (mainly glucose) and with the enzymes used for the pre-digestion itself. A dialysis step with low MW cut-off (2000) was used to remove the digestion products before fermentation. However, low MW pyrodextrins, which might be non-digestible and fermentable, were also lost during this step. Consequently, pyrodextrins used in this work had MW in the range of 2000 to 105,000.

Pyrodextrins do not belong to any of the three categories of resistant starch originally proposed (Englyst *et al.*, 1992). Some authors have suggested new

categories of resistant starch to include not only chemically modified starches (Brown *et al.*, 1995), but also physically modified starches (like extruded starch) and amylo-lipid complex (Asp, 1997). Since pyrodextrins have different glucosidic bonds than those present in native starches, they may belong to the category that includes chemically modified starches.

There was an almost complete fermentation of all starch samples under the fermentation conditions used (Edwards *et al.*, 1996) based on net total SCFA production, fall in pH, starch and carbohydrate content before and after 24 h anaerobic incubation (Table 3.1). Net total SCFA and pH for pre-digested native starches were very similar to those reported elsewhere for raw potato starch (Edwards *et al.*, 1996). Although raw and native starches are not part of the western diet, they were used here for comparison purposes. The heat-acid treatment did not change the production of gases in the faecal cultures.

Two approaches were used to assess the amount of substrate consumed during fermentation. An enzymatic, highly specific approach using Englyst method, as described by Edwards *et al.* (1996), was used for the pre-digested native starches. On the other hand, a more general, chemical approach using a modification of the anthrone-sulphuric acid assay was used for the pre-digested pyrodextrins. These methods differ in their specificity and they were used because the Englyst method underestimated the starch content of pyrodextrins, as shown under "Total starch" on Table 3.2. This was possibly due to the presence of non-starch linkages in such modified starches, which cannot be hydrolysed by the enzymes used in the assay.

Regarding the SCFA profile, pre-digested pyrodextrin fermentation showed the high proportion of butyrate that characterises native starch fermentation (Edwards

& Rowland, 1992), yet there was an increase in the molar ratio of propionate with a parallel decrease in acetate. Another study (Cummings *et al.*, 1996) has shown a lower propionate ratio in raw potato and banana starches (resistant starch type 2) when compared with both wheat starch (digestible starch) and retrograded wheat and corn starches (resistant starch type 3). However, differences seen in the present study were 2- to 6.5-fold larger than those previously reported (Cummings *et al.*, 1996). The drop in pH was higher for the pre-digested native starches than for pyrodextrins (Table 3.1). This may be due to the higher proportion of acetate, which has a lower pKa (4.74) than the other SCFA, present in the native sample cultures.

It is interesting to note that the only carbohydrate moiety present in pyrodextrins is glucose. The changes during pyrodextrinisation of native starches yield oligo- or polysaccharides with lower MW than native starches, along with the new glucosidic bonds. The extent of these changes depends on the conditions employed (Wurzburg, 1986a), but usually render highly branched (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b; Ohkuma & Wakabayashi, 2001), water soluble, resistant-to-digest pyrodextrins (Laurentin *et al.*, 2003). It is not clear why pre-digested pyrodextrins had a higher propionate production than their pre-digested native starches. However, it could be due to an increased solubility and/or presence of non-starch bonds caused by transglucosidation reactions. The presence of these new, non-starch bonds may change the starch structure in a way that modifies the accessibility and/or affinity of the bacterial enzymes to the bonds.

High solubility, on the other hand, may also be important. α -Glucooligosaccharide (branched pentasaccharide with 1→2, 1→4 and 1→6 α -bonds) and maltodextrin-like oligosaccharides (branched oligosaccharides with 2000 MW and 1→4, 1→6, 1→2 and 1→3 α - and β -bonds), both highly soluble and resistant-to-digest carbohydrates yielded similar SCFA molar ratio to the pre-digested pyrodextrins (Table 3.3) when fermented with human faecal microflora *in vitro* (Flickinger *et al.*, 2000).

Table 3.3. Short-chain fatty acid proportion for several glucose-based carbohydrates in *in vitro* cultures of human faeces or in human faecal associated rats *in vivo*.

Carbohydrate	SCFA proportion* (%)			Soluble in water	Glucosidic bond [†]	Reference
	Ac	Pr	Bu			
Glucose	64	22	14	yes	None	This thesis
α -Gluco-oligosaccharide	60	24	16	yes	α	(Flickinger <i>et al.</i> , 2000)
Maltodextrin-like oligosaccharide	62	30	8	yes	α and β	(Flickinger <i>et al.</i> , 2000)
Pre-digested pyrodextrin [‡]	56	27	17	yes	α and β	This thesis
Pre-digested native starch [‡]	70	13	16	no	α	This thesis
Cellulose [§]	41	52	7	no	β	(Velázquez <i>et al.</i> , 2000)
Oat husk	65	26	9	no	β	(Roland <i>et al.</i> , 2002)
Oat bran [¶]	56	22	22	no	β	(Karppinen <i>et al.</i> , 2000)

SCFA, short-chain fatty acids; Ac, acetate; Pr, propionate; Bu, butyrate.

* Mean values in molar ratio based on net total SCFA (Ac + Pr + Bu).

[†] Conformation of glucosidic bonds.

[‡] Mean data from this thesis.

[§] From Sigma (St. Louis, MO, USA).

^{||} Mainly cellulose and hemicellulose + pectin; in human faecal flora associated rats.

[¶] Mainly β -glucan.

However, insoluble β -bonded fibres also show a SCFA profile similar to the pre-digested pyrodextrins. A molar ratio of 65:26:9 for acetate, propionate and butyrate, respectively, was reported when oat husk (44 % cellulose, 50 % hemicellulose + pectin) was used as fibre source in a diet for rats inoculated with human faecal flora (Roland *et al.*, 2002). Oat bran (mainly β -glucan) (Karppinen *et al.*, 2000) and cellulose (Velázquez *et al.*, 2000) fermentation *in vitro* also yielded a high propionate molar ratio (Table 3.3). Acid catalysed pyrodextrins, as prepared in this study, are thought to contain a significant proportion of β -bonded glucosidic linkages, as shown for wheat starch heated during 4 h at 180 °C without addition of any catalyst (Siljeström *et al.*, 1989). However, the presence of such β -bonds in acid catalysed pyrodextrins has yet to be confirmed. Our attempt to look for and quantify such bonds in the starch pyrodextrins, prepared as described by Ghali *et al.* (1979), is described in the next chapter.

In conclusion, pyrodextrinisation of starches isolated from potato, lentil and cocoyam resulted in the production of a soluble material that escapes digestion, but was extensively fermented *in vitro* by the colonic bacteria of healthy adults. This fermentation is characterised by higher proportions of propionate and lower proportions of acetate than the native starch, although the high proportion of butyrate characteristic of starch fermentation was maintained. It is not yet clear what causes such changes in SCFA molar ratios.

However, it has been shown that propionate decreased lipogenesis in isolated rat hepatocytes (Nishina & Freedland, 1990) and it has been suggested that colonic propionate utilisation in the liver may decrease lipogenesis (Wolever *et al.*, 1995) and cholesterol (Wolever *et al.*, 1991) synthesis from colonic acetate. More

research is needed to see if ingestion of pyrodextrins can have this effect in humans. Indeed, we designed a human trial to study whether Fibersol-2, a commercial pyrodextrin, can change the *in vivo* faecal SCFA profile in a similar way as did the pyrodextrins studied in this thesis in the *in vitro* model of the human colon. Moreover, we attempted to investigate whether those changes in the production of SCFA could reduce postprandial lipid in healthy adults. More details of these studies are given in Chapter 6.

The study described in this chapter was published in the *European Journal of Nutrition* (Laurentin & Edwards, 2004) (see Appendix 2).

Chapter 4.

Insight into the starch pyrodextrin structure using nuclear magnetic resonance spectroscopy

This chapter describes an attempt to characterise the chemical structure of starch-pyrodextrins using nuclear magnetic resonance spectroscopy (NMR) as a tool.

4.1. Introduction

Starch in its native form is composed of glucose moieties linked by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glucosidic bonds as shown in Figure 1.1 (for amylose) and Figure 1.2 (for amylopectin). Starch pyrodextrins, on the other hand, undergo chemical modifications by the action of heat, usually in the presence of catalysts. These modifications occur mainly because of hydrolysis and transglucosidation reactions as discussed in Chapter 1. The prevalence of either reaction depends on the conditions used during pyrodextrinisation. In fact, different combinations of the starch/water content, acidity of the preparation, temperature and time of pyroconversion produce a variety of pyrodextrins, which have been put together into three categories; white dextrins, yellow dextrins and British gums (Evans & Wurzburg, 1967; Wurzburg, 1986a). White dextrins (prepared in an acidic environment and low temperature) mostly have a lower molecular weight than the starch from which they are made. On the other hand, yellow dextrins (also prepared in an acidic environment, but at high temperature) and British gums (prepared in a neutral-to-basic pH and high temperature) have a lower molecular weight, higher degree of branching and contain bonds not present in the native starches.

One of the first studies of the structure of pyrodextrins was reported by Brimhall (1944). She provided a semiquantitative picture of a commercial pyrodextrin prepared in the absence of an acid catalyst. The average degree of polymerisation of this pyrodextrin, estimated by methods based on the reducing power, was 66 glucose residues (ca. 10,000 MW). Methylation experiments indicated the presence of 4 or 5 short branches and hydrolysis with β -amylase suggested that each branch had approximately 5 glucose units (Brimhall, 1944). Years later, Kerr & Cleveland (1953) produced a pyrodextrin by heating corn amylose at 175 °C, without any catalyst, for up to 5 hours. They observed a drop in the average degree of polymerisation from 235 to 58 glucose residues in the first hour of pyroconversion. The increase in the degree of branching was suggested by a drop in the proportion (from 95 % to 15 % after 5 h) of the material that can be hydrolysed to maltose by the action of β -amylase and a decrease in the intensity of the blue colour developed after complexation with iodine (Kerr & Cleveland, 1953).

The presence of non-starch bonds in the pyrodextrin structure was suggested by Geerdes *et al.* (1957) using a commercial acid-converted corn pyrodextrin and methylation analysis. In this approach, methyl groups can be attached to the hydroxyl groups of the glucose residues that are not involved in glucosidic bonds in the polymer. Then, the methylated polymer is broken down by acid-hydrolysis, the monomers can be separated, identified, quantified by chromatographic and other chemical techniques and the glucosidic bonds identified by inference. The methylation analysis of native starch, for instance, produces three different methylated derivatives: 2,3,4,6-tetra-, 2,3,6-tri- and 2,3-di-O-methyl-D-glucose, corresponding to the glucose residues at the non-reducing end, the internal

residues (with the (1→4) bonds) and the branching points (with the (1→4) and (1→6) bonds) of the starch, respectively. In their work, Geerdes *et al.* (1957) found not only the three methylated sugars derived from the starch (accounting for 80 % of the methylated residues), but also seven additional methylated sugars derived from the occurrence of the non-starch linkages. The most abundant of these identified components was 2,6-di-O-methyl-D-glucose (abundance 10 %), which corresponds to a branching point with (1→4) and (1→3) glucosidic bonds. Methylated residues that suggest the presence of (1→2) bonds were also found.

Further confirmation of the existence of the non-starch bonds was reported by Thompson & Wolfrom (1958) using a pyrodextrin prepared by heating amylose at 185 – 200 °C, without an acid catalyst. They used a fragmentation approach in which the pyrodextrin was submitted to a partial acid hydrolysis and the products were isolated and identified by chromatography and others chemical methods. In this way, they found important amounts of maltose, isomaltose, gentiobiose and sophorose as hydrolysis products, suggesting the presence of $\alpha(1\rightarrow4)$, $\alpha(1\rightarrow6)$, $\beta(1\rightarrow6)$ and $\beta(1\rightarrow2)$ bonds, respectively, in the pyroconverted amylose.

More recently, Siljeström *et al.* (1989) characterised the non-digestible fraction (obtained after hydrolysis with Termamyl and amyloglucosidase) of a wheat pyrodextrin prepared by heating the starch at 180 °C for 4 hours in the absent of any catalyst. The methylation analysis showed the presence of at least (1→3) bonds. As the methylation analysis does not provide information about the anomeric configuration of the glucosidic bonds, Siljeström *et al.* (1989) used ¹H-NMR spectroscopy to investigate so. They could not confirm the presence of the $\beta(1\rightarrow3)$ bond in the non-digestible fraction of the wheat pyrodextrin, but they

did detect $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ bonds; linkages that can be overlooked by the methylation analysis.

More than a decade ago, Matsutani Chemical Industries in Japan developed and commercialised Fibersol-2, a product made of the non-digestible fraction of a corn pyrodextrin produced in the presence of an acid catalyst. During the development of Fibersol-2, this company patented the production of different non-digestible pyrodextrins² from both potato (Ohkuma *et al.*, 1993a) and corn (Ohkuma *et al.*, 1993b) starches. The structure of these non-digestible pyrodextrins was studied using the methylation analysis and some of their results are summarised here to highlight the effect of different pyroconversion conditions and starch sources on the degree of branching and the proportion of $(1\rightarrow3)$ and $(1\rightarrow2)$ bonds (Table 4.1). For instance, the degree of branching of potato pyrodextrins was high and very similar in all of the conditions whereas corn pyrodextrins showed different degree of branching depending on the pyroconversion conditions. Corn pyrodextrins, on the other hand, had a higher proportion of $(1\rightarrow3)$ and $(1\rightarrow2)$ bonds than potato pyrodextrins. The presence of these two non-starch bonds was different in condition 3 when compared with the other conditions (Table 4.1). These Japanese researchers claimed that the $(1\rightarrow3)$ linkage was the most abundant non-starch bond in the non-digestible pyrodextrins they developed.

² The Matsutani group uses the generic terms "indigestible dextrin" or "resistant maltodextrin" to refer to their commercialised products. As it was pointed out in Chapter 1, the use of the term "dextrin" may mislead the reader because it refers to a wide group of molecules. Hence, "non-digestible pyrodextrin" or "resistant pyrodextrin" will be used in this thesis as generic names for those pyrodextrins that have been hydrolysed with amylases to enrich their non-digestible components.

Table 4.1. Degree of branching and proportion of non-starch bonds in non-digestible pyrodextrins prepared at different conditions.

Condition	Tem- perature (°C)	Time (min)	HCl (g/kg starch)	Proportion (%) of glucose residues*			
				at the non-reducing end [†]		with (1→3) and (1→2) bonds [‡]	
				Potato	Corn	Potato	Corn
1	150	55	1.00	27	22	17	29
2	165	40	0.75	28	22	18	28
3	180	25	1.20	26	27	23	24

* Data calculated from methylation experiments of non-digestible pyrodextrins (the digestible fraction was hydrolysed using Termamyl and glucoamylase) (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b).

[†] The proportion of 2,3,4,6-tetra-O-methyl-D-glucose derivative was used as an indicator of the degree of branching because this derivative is produced from the residues at the non-reducing end of the chain.

[‡] The percentage of (1→3) and (1→2) bonds was the sum of 2,4,6-tri-O-methyl- and 2,6-di-O-methyl-D-glucose derivatives for the (1→3) bond, 3,6-di-O-methyl-D-glucose derivative for the (1→2) bond and other minor methyl glucose derivatives reported (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b).

Using pyrodextrins from different starch sources, prepared under the same conditions as in this thesis, Laurentin *et al.* (2003) showed that an extensive depolymerisation occurred during the pyroconversion process. These pyrodextrins did not show any endotherms due to gelatinisation (measured by differential scanning calorimetry) or any detectable viscosity up to 80 g/L (measured by rapid visco-analyser). Moreover, gel filtration chromatography showed that these pyrodextrins are a complex mixture of molecules, all with apparent MW below 105,000. For instance, corn pyrodextrin had four main peaks of apparent MW at 5000, 10,000, 19,000 and 40,000 whereas lentil pyrodextrin showed three main peaks at 5000, 14,000 and 40,000 apparent MW (Laurentin *et al.*, 2003).

In 1999, I proposed a hypothetical structure for these starch pyrodextrins after their physicochemical characterisation (Laurentin, 1999; Laurentin *et al.*, 2003) and using the results of the methylation studies carried out by the Matsutani group

(Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b). This structure is shown in Figure 4.1. More recently, the Japanese group have proposed a very similar structure for their commercial pyrodextrin, Fibersol-2 (Ohkuma & Wakabayashi, 2001).

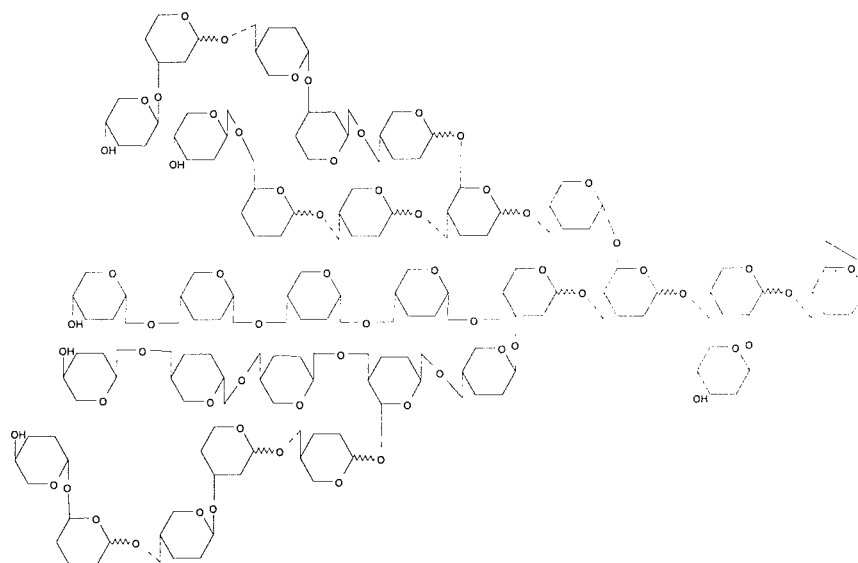


Figure 4.1. Hypothetical structure of pyrodextrins.

Glucose residues are shown without the primary and secondary hydroxyl groups, except those for the non-reducing end, which are represented with the hydroxyl group attached to C4. Note that 1,6-anhydro- β -D-glucose is at the other end of the molecule. Taken from Laurentin (1999).

The evidence presented about the structure of pyrodextrins produced at high temperature with (yellow dextrins) and without (British gums) acid catalysts, picture highly branched, low molecular weight molecules, with at least (1 \rightarrow 2) and (1 \rightarrow 3) bonds for yellow dextrins and β (1 \rightarrow 2), β (1 \rightarrow 4) and β (1 \rightarrow 6) bonds, along with the (1 \rightarrow 3) bond, for British gums. There is therefore a lack of information concerning the anomeric conformation of the glucosidic linkages in the acid converted pyrodextrins. The chemical mechanism postulated by Lowary & Richards (1991) and discussed in Chapter 1, predicts the presence of β linkages in these pyrodextrins.

Hence, the main aim of this work was to search for the presence of β -bonds in a potato pyrodextrin, prepared under the conditions reported by Ghali *et al.* (1979), using NMR spectroscopy. The secondary aims were to compare the structure of pyrodextrins prepared from different starch sources and to investigate the effect of loss of small molecules (< 2000) by dialysis on the structural features of starch pyrodextrins as seen on NMR spectroscopy.

4.2. Methods

4.2.1. NMR spectroscopy

All samples were dissolved in deuterium oxide (D_2O) (Aldrich Chemical Co., Milwaukee, WI, USA; isotopic purity: 99.9 atom % D) at an appropriate concentration (specified below). Spectra were obtained using a DPX400 spectrometer, which was operated by Dr David S. Rycroft (NMR Spectroscopy Laboratory, Department of Chemistry, University of Glasgow). Both 1H NMR (at 400 MHz) and ^{13}C NMR (at 100 MHz, proton-decoupled) spectra show chemical shift (δ) values reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Acetone and TMS were used as external and internal references, respectively. 1H NMR spectra were parallel-shifted to give the signal of the residual solvent (HOD) a value of 4.70 ppm. ^{13}C NMR spectra were also parallel-shifted to give C6(i) atom a value of 61.6 ppm, in agreement with its normal shift (Bock *et al.*, 1984). As standard practice in the NMR Spectroscopy Laboratory, a 1H NMR spectrum was run before running the ^{13}C NMR spectrum to check the quality of the deuterated water. All runs were carried out at 25 °C, using a 5 mm dual probe.

4.2.2. Chemical shift assignment

Potato pyrodextrin was used as a model for the assignment of chemical shifts. Solutions of 10 and 100 g/L were used for ^1H and ^{13}C NMR spectroscopy, respectively. The chemical shift assignment was performed using an empirical approach with values previously reported in the literature. Advice was sought from Dr Michael C. Jarvis (Department of Chemistry, University of Glasgow) for the interpretation of spectra.

4.2.3. Fingerprint ^1H NMR spectra

^1H NMR spectroscopy was used to compare the chemical structure of the pyrodextrins prepared as described in Chapter 2 (Ghali *et al.*, 1979). Cocoyam, corn, potato and lentil pyrodextrins were used (10 g/L solution). For comparison purposes, a commercial soluble starch (Reidel-de Hagaën, Gillingham, UK) at 10 g/L was also included. The commercial starch was dissolved by water bath incubation at 95 °C for 15 min with frequent vortex mixing.

4.2.4. Dialysis of starch pyrodextrin

As the *in vitro* fermentation of starch pyrodextrins (Chapter 3) was performed by using pre-digested samples, which were dialysed to remove the digestion products, lentil pyrodextrin (1 g) was dissolved in 10 mL water and dialysed against water at 4 °C, using a dialysis bag (Sigma-Aldrich Company Ltd., Dorset, UK) with 2000 MW cut-off as described in Chapter 2. The retentate was freeze-dried (Edwards freeze dryer Micro Modulyo) and milled in a mortar. A solution in D_2O (100 g/L) was used for ^{13}C NMR spectroscopy to investigate the effect of such a dialysis step.

4.2.5. Notation of hydrogen and carbon atoms

To notate unambiguously the different protons and carbon atoms, a notation system was adapted from the one used by Nilsson *et al.* (1996). The glucose residues in pyrodextrins were divided into four groups: those involved in linear $\alpha(1\rightarrow4)$ linkages in the main chain (namely internal residues), those involved in terminal non-reducing ends, those involved in terminal reducing end and those involved in branching points (either α - or β -bonds). In this notation, it is assumed that both terminal non-reducing and reducing ends are attached only to the internal residues by $\alpha(1\rightarrow4)$ bonds in agreement with the known structure of amylopectin.

The carbons in a glucose internal residue are denoted by the addition of (i) for instance, C1(i); those in a terminal non-reducing end are followed by (t) for instance, C4(t); those in the reducing end are followed by (α -r) or (β -r) depending on their anomeric configuration for instance, C1(α -r) or C2(β -r); and those in a branching point are followed by the corresponding bond (for instance, C1 $\beta(1\rightarrow2)$ and C2 $\beta(1\rightarrow2)$ denote the two carbons in $\beta(1\rightarrow2)$ glycosidic bond. The protons were denoted in a similar way as the carbons, for instance: H1(i), H4(t), H1(β -r) and H1 $\alpha(1\rightarrow6)$.

4.3. Results

Potato pyrodextrin was used as a model to identify the different resonance signals due to both ^1H and ^{13}C NMR spectroscopy. In this section, I describe the process followed to identify the resonance signals, first from the ^1H NMR and then from the ^{13}C NMR spectroscopy. Due to the nature of this work, where the chemical shift assignment was done by comparing the experimental NMR signals with data from the literature, the assignments will be discussed in this section.

^1H NMR spectra are relatively easier to obtain than those for ^{13}C NMR. ^1H is the most naturally abundant isotope of hydrogen; therefore, a low number of scans are needed to obtain a spectrum. For this thesis, for instance, the number of scans used was only 16, making the whole run last for a few minutes. On the other hand, the isotope ^{13}C counts for only 1.1 % of the naturally occurring carbon (Morrison & Boyd, 1992) and, therefore, a very large number of scans were needed to overcome the low signal-noise ratio. For this thesis, 32,000 scans were usually used for each spectrum and that represented a very long running time (about 9 hours). Hence, the use of NMR spectroscopy of ^{13}C , even though it can be more informative than that of ^1H (as discussed below), in this thesis was limited.

4.3.1. Proton NMR spectroscopy for potato pyrodextrin

The ^1H NMR spectrum for potato pyrodextrin in D_2O (10 g/L) is shown in Figure

4.2. To identify the resonance signals, I compared the spectra from different carbohydrates (glucose, anhydro-glucose, maltose, cellobiose, lactose, melibiose, gentiobiose, maltotriose, maltotetraose, maltohexaose and maltoheptaose) catalogued in “The Aldrich library of ^{13}C and ^1H FT NMR spectra” (Pouchert & Behnke, 1993). These spectra were compared to identify as many structural features as possible and then the identified features were located in the potato pyrodextrin spectrum. However, difficulties arose when the sharp signals of the low molecular weight saccharides were compared with the broader peaks of higher molecular weight compounds like the potato pyrodextrin.

Moreover, the interpretation of the spectrum was further hampered because of the multiple peaks associated with different sets of equivalent hydrogen atoms. This phenomenon of multiple absorptions is common in NMR spectroscopy and is known as spin-spin splitting. It is produced by the interaction of the nuclear spins

of one or more neighbouring hydrogen atoms with that particular set of equivalent protons (Morrison & Boyd, 1992). In other words, the magnetic field “felt” by each hydrogen nucleus is affected by the small magnetic fields of the other hydrogen nuclei in the vicinity. The proton attached to C1 in glucose (H1), for instance, has one neighbouring proton at C2 (H2), which can be spinning in either of two possible directions. In those glucose molecules where H2 is spinning clockwise, H1 experiences a magnetic field that differs slightly from the magnetic field felt in those glucose molecules where H2 is spinning counter clockwise (the difference between these two magnetic fields is expressed as the coupling constant, J). Hence, two slightly different external magnetic fields are needed for H1 in all glucose molecules to absorb the electromagnetic radiation. Two resonance signals, therefore, are produced. These two peaks are called a doublet.

A similar effect is produced by H1 on H2; now H1 is the neighbouring proton of H2 and its spinning affects the magnetic field experienced by H2. In general, sets of hydrogen atoms in neighbouring carbon atoms couple with each other.

However, the case for H2 is more complicated. Its signal is not a doublet, but a triplet because H2 has another neighbouring proton at C3 (H3), which affects (together with H1) the magnetic field experienced by H2. In general, a set of one or more protons that has n equivalent neighbouring protons has $n + 1$ peaks in the NMR spectra (Morrison & Boyd, 1992). For a glucose molecule, for instance, doublets are produced by H1 and H6; triplets are produced by H2, H3 and H4; and a quartet is produced by H5. This multiplicity of the resonance signals may overlap signals from different hydrogen atoms. In addition, the multiplicity may disappear when the molecular weight increases (Gidley, 1985), making the spectrum interpretation even more complex. An example of these problems is

illustrated in the chemical shift range from 4.0 to 3.4 ppm in Figure 4.2, where most of the major structural features of the spectra were fused together which made them impossible to resolve.

In spite of all these limitations, several minor features were identified in potato pyrodextrin ^1H NMR spectrum (Figure 4.2) and summarised in Table 4.2.

Comparison of the resonance signals of α -D-glucose with those for maltose, maltotriose, maltotetraose, maltohexaose and maltoheptaose (Pouchert & Behnke, 1993) revealed that the signal at 5.4 – 5.3 ppm was present in the oligomers but not in the monomer. This signal therefore may correspond to the hydrogen atom attached to the anomeric carbon involved in the $\alpha(1\rightarrow4)$ bond, i.e. H1(i) and H1(t). Potato pyrodextrin had a broad peak in that region ($\delta = 5.33$ ppm) that was assigned to H1(i) and H1(t). The spectra for melibiose (6-O-(α -D-galactopyranosyl)-D-glucopyranose) has a signal between 4.95 – 4.85 ppm that it is not present in any other of the carbohydrates compared (Pouchert & Behnke, 1993). This peak is more likely to be the resonance signal for the H1 in the $\alpha(1\rightarrow6)$ bond. Potato pyrodextrin showed a broad and small peak in this region ($\delta = 4.89$ ppm) and it was assigned to H1 $\alpha(1\rightarrow6)$ (Figure 4.2). These two features represent the protons attached to the anomeric carbons in the two linkages found in the starch polymers. Further support for these assignments was found in the literature. Gidley (1985), using panose (α -D-glucopyranosyl unit attached at C6 of non-reducing moiety of maltose) as a simple model for starch, identified two doublets at 5.40 and 5.00 ppm and assigned them to H1(i) and H1 $\alpha(1\rightarrow6)$, respectively (Table 4.2, Ref. A column). Similarly, Nilsson *et al.* (1996) assigned

δ values of 5.35 and 4.94 ppm to these structural features using potato starch (Table 4.2, Ref. B column).

Glucose, maltose, maltose-oligomers, lactose, cellobiose, gentiobiose and melibiose—all reducing carbohydrates—show two peaks (in some cases, doublets) at 5.2 – 5.1 and 4.6 – 4.5 ppm (Pouchert & Behnke, 1993) that correspond to the resonance of the two anomeric configurations of the H1 in the reducing end. Potato pyrodextrin had a small doublet at 5.15 ppm that was assigned to H1(α -r). However, the resonance of the H1(β -r) was not assigned because several small signals were overlapped in this region of the spectrum (4.6 – 4.5 ppm; Figure 4.2). Panose also has these two signals assigned to the anomeric protons (Table 4.2, Ref. A column).

Two other small structural features present in the starch polymers can be identified by ^1H NMR spectroscopy. One triplet at 3.4 – 3.3 ppm, that is clearly resolved in maltose and its oligomers yet not in glucose (Pouchert & Behnke, 1993), corresponds to H4(t) (Nilsson *et al.*, 1996). This triplet was clearly resolved in potato pyrodextrin (δ = 3.34 ppm, Figure 4.2). The other feature was a triplet located slightly upfield (3.2 – 3.1 ppm) of H4(t), present in glucose, maltose and its oligomers (Pouchert & Behnke, 1993) and assigned to H2(β -r) (McIntyre & Vogel, 1990). This signal did not appear in potato pyrodextrin.

In addition to these structural features, which are also present in the starch polymers (Nilsson *et al.*, 1996), potato pyrodextrins showed proton resonance that may be attributable to the presence of 1,6-anhydro- β -D-glucose and non-starch bonds. The ^1H NMR spectrum for 1,6-anhydro- β -D-glucose shows three resonance peaks of interest at 5.5 – 5.4, 4.6 – 4.5 and 4.1 – 4.0 ppm (Pouchert & Behnke,

1993). It is likely that two of these signals represent the resonance of the protons involved in the intramolecular $\beta(1\rightarrow6)$ bond of the anhydro-glucose. The peak at 5.5 – 5.4 ppm could correspond to H1(a) and the peak at 4.1 – 4.0 ppm could correspond to H6(a). Both peaks are downfield to the corresponding H1 and H6 from glucose (Pouchert & Behnke, 1993). Potato pyrodextrin showed a small peak in the 5.5 – 5.4 ppm region ($\delta = 5.39$ ppm) that could be assigned to H1(a) (Table 4.2). Although there were some signals at 4.1 – 4.0 ppm, it was not possible to differentiate them from the signals due to other β bonds (see below). Finally, there were also signals at 4.6 – 4.5 ppm, but it was not possible to resolve them from those belonging to H1(β -r) (Figure 4.2).

Spectra for disaccharides were used as models for the β bond resonance peaks. Unfortunately, ^1H NMR spectra for only two out of the four possible β bonds were found in the literature. In both lactose (4-O-(β -D-galactopyranosyl)-D-glucopyranose) and cellobiose (4-O-(β -D-glucopyranosyl)-D-glucopyranose), the peak for the H1 involved in the glycosidic link is more likely to be the doublet at 4.4 – 4.3 ppm for lactose and at 4.5 – 4.4 ppm for cellobiose. Both doublets are upfield of the signal for H1(i) in maltose (5.5 – 5.4 ppm) (Pouchert & Behnke, 1993) as expected by the *anti* conformation of H1 and H2 in the $\beta(1\rightarrow4)$ bond. On the other hand, gentiobiose (6-O-(β -D-glucopyranosyl)-D-glucopyranose) had two signals at 4.5 – 4.3 and 4.2 – 4.0 ppm that are not present in the maltose or melibiose spectra (Pouchert & Behnke, 1993). These two signals are probably the resonance of the H1 and H6 in the $\beta(1\rightarrow6)$ bond. Potato pyrodextrin showed small peaks in these two regions of the spectrum, but it was not possible to assign any of these signals because of overlapping (Figure 4.2). It is noteworthy to mention that

several small peaks (some of them doublets) were present between 4.5 and 4.3 ppm and those signals may correspond to the H1 in the different β bonds. Similarly, the small peaks between 4.2 and 4.0 ppm may correspond to the H6 in both $\beta(1\rightarrow6)$ link and 1,6-anhydro- β -D-glucose (Table 4.2). Finally, the ^1H NMR spectrum for potato pyrodextrin had two small, but clearly resolved structural features: one doublet at 5.1 – 5.0 and one triplet at 3.5 – 3.4 ppm (Table 4.2). The identity of these hydrogen atoms is not known at present.

4.3.2. Carbon-13 NMR spectroscopy of potato pyrodextrin

The ^{13}C NMR spectrum for potato pyrodextrin in D_2O solution (100 g/L) is shown in Figure 4.3. Chemical shift values from the literature (Ritchie *et al.*, 1976; Bock *et al.*, 1984) were used to identify most of the signals from this spectrum and the results are shown in Table 4.3.

NMR spectroscopy of ^{13}C has certain advantages over that of ^1H . The very low natural abundance of the carbon isotope, although inconvenient when a high signal-noise ratio is required (as in this thesis), is useful for eliminating the spin-spin coupling signal from neighbouring carbons. The probability that two ^{13}C isotopes are present in the same molecule is very low, even lower if we consider that they have to be next to each other ($-\text{C}-^{13}\text{C}-^{13}\text{C}-\text{C}-$) to affect their magnetic fields and produce splitting. ^{13}C NMR spectra, therefore, does not have multiplicity due to carbon atom spin-spin coupling. However, protons linked to each carbon atom can produce spin-spin splitting. Indeed, $^{13}\text{C}-^1\text{H}$ coupling patterns give information about the number of hydrogen atoms linked to each carbon atom and may help for signal assignment (Ritchie *et al.*, 1976). However, with the appropriate instrumentation, it is possible to eliminate the multiplicity due to the presence of hydrogen atoms. This technique, called proton-decoupled

^{13}C NMR, was used in this thesis. We expected, therefore, that each carbon atom in potato pyrodextrin had one peak.

Another advantage of ^{13}C NMR is that there is more information in the literature regarding the chemical shift assignments of low molecular weight carbohydrates (Ritchie *et al.*, 1976; Bock & Pedersen, 1983; Bock *et al.*, 1984). In fact, some generalisations have been recognised and summarised as follows: resonance at lowest field (between 110 and 90 ppm) are attributable to the anomeric carbon atoms (C1) in pyranoses and furanoses, while resonance at highest field (between 64 and 60 ppm) to carbon atoms carrying primary hydroxyl groups (C6) (Bock & Pedersen, 1983). In addition, carbon atoms bearing secondary hydroxyl groups give signals between 85 and 65 ppm (Bock & Pedersen, 1983), with the carbon atoms involved in the glycosidic linkages (except for 1→6 bonds) resonate between 88 and 79 ppm (Usui *et al.*, 1973). This information can be used as a first step to identify signals from higher molecular weight carbohydrates, like pyrodextrins. This approach, therefore, was used in this thesis to interpret the ^{13}C NMR spectra.

Resonance signals from the carbons belonging to both the non-reducing and the reducing ends as well as the internal moieties of potato pyrodextrin were identified using maltopentose and maltoheptaose as models (Bock *et al.*, 1984). The major features shown in Figure 4.3 corresponded to the resonance of carbon atoms belonging to the internal residues. These residues are the most abundant and constitute the backbone of the starch polymers; therefore, they are likely to be also abundant in pyrodextrins. There was good agreement (i.e. chemical shift values did not differ by more than 0.1 ppm) for all carbons, except C1(i), C3(i)

and C4(i) (Table 4.3 Ref. A column). These differences, especially for C4(i), may be explained by the free rotation capacity of the $\alpha(1\rightarrow4)$ bond. The signals for the glucose moieties in the non-reducing end of the pyrodextrin also had good agreement with those reported by Bock *et al.* (1984), except for C4(t) and C5(t). It is interesting that there were at least three peaks at 70.8 – 70.5 ppm (Figure 4.3). The largest of these peaks was at 70.5 ppm, which may correspond to C4(t). The shifts for the two possible anomeric conformations of glucose residues at the reducing end were found at 96.9 and 93.1 ppm, for the β and α anomers, respectively. Some other signals for carbon atoms at the reducing end were also possible to identify (Figure 4.3) and the chemical shift values are given in Table 4.3.

The monomer anhydro-glucose (1,6-anhydro- β -D-glucopyranose) was used to locate its resonance signals (Ritchie *et al.*, 1976). The potato pyrodextrin spectrum had six clearly defined peaks (δ values in Table 4.3) that may correspond to the anhydro-glucose. However, all the six signals were shifted downfield by 0.5 (SD 0.1) ppm when compared with those reported by Ritchie *et al.* (1976). The Aldrich library (Pouchert & Behnke, 1993) also reports six peaks whose δ values can be parallel-shifted with our experimental results. It is interesting that all six resonance signals were found, because 1,6-anhydro- β -D-glucose has been proposed to be present at the polymer reducing end after the transglucosidation reactions (Thompson & Wolfrom, 1958; Theander & Westerlund, 1987) that take place during the starch modification. If this anhydro-glucose were at the terminal reducing end of the pyrodextrin polymers, a downfield chemical shift (8 – 11 ppm) should be seen for C4(a) (Usui *et al.*, 1973). This effect, called the

α -effect, can be illustrated by comparing C4(t) against C4(i) δ values for potato pyrodextrin (Table 4.3). When C4 had the hydroxyl group attached —like in C4(t)—, its chemical shift was 70.8 – 70.5 ppm, but when C4 had a glucose residue directly attached —like in C4(i)—, its shift was 77.9 ppm. The presence of the glucosidic bond deshielded the C4 nucleus and, therefore, its resonance appeared between 7.1 and 7.4 ppm downfield. The 1,6-anhydro- β -D-glucose resonance signals did not show the α -effect; therefore, the anhydro-glucose produced during the starch pyroconversion (or at least some of them) may have broken off from the polymer and stayed as monomers.

Several disaccharides (Bock *et al.*, 1984) were used as models for the carbon atoms linked by the branching bonds. The selected disaccharides were those with the α anomer configuration in the aglycone residue because it was assumed that all branching points were part of the polymer backbone, i.e. aglycone groups in the branching points had $\alpha(1\rightarrow4)$ bonds at both C1 and C4, in agreement with the known structure of β -limit dextrins. Hence, one drawback of using disaccharides here was that none of them has such $\alpha(1\rightarrow4)$ bonds. In spite of this, some information could be achieved. Isomaltose (6-O-(α -D-glucopyranosyl)-D-glucopyranose), for instance, was used as the model for the $\alpha(1\rightarrow6)$ bond (Bock *et al.*, 1984). Potato pyrodextrin had two short peaks at 98.9 and 66.3 ppm that could be assigned to C1 $\alpha(1\rightarrow6)$ and C6 $\alpha(1\rightarrow6)$, respectively.

Sophorose (2-O-(β -D-glucopyranosyl)-D-glucopyranose), laminaribiose (3-O-(β -D-glucopyranosyl)-D-glucopyranose), cellobiose and gentiobiose were used as the models for $\beta(1\rightarrow2)$, $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ bonds, respectively (Bock *et al.*, 1984). The resonance for the anomeric carbon atoms involved in the

β bonds seemed to be located at 106 – 103 ppm (Figure 4.3). The most downfield peak was clearly resolved, but the other three were broad and very close to each other. All four signals were, as the signals for anhydro-glucose, shifted 0.65 (sd 0.06) ppm downfield when compared with the values reported by Bock *et al.* (1984) (Table 4.4). On the other hand, three of the signals for the carbon atoms in the aglycone residue were located at 82 – 78 ppm and the other was upfield at 70 – 69 ppm. Based on the chemical shift differences between the signals reported in the literature and the height of the peaks, it was possible to make a tentative assignment of these bond atoms. The δ values are summarised in Table 4.4.

4.3.3. Fingerprint ^1H NMR spectra

^1H NMR spectroscopy was used as a quick way to try to compare the chemical changes due to pyrodextrinisation on several starch sources. Spectra from pyrodextrins produced from cocoyam, corn, potato and lentil, along with the spectrum for soluble starch, are shown in Figure 4.4. All pyrodextrins had the same structural features described in section 4.3.1 for potato pyrodextrin (Table 4.2). Several overlapping small peaks between 4.5 and 4.0 ppm were present in all the pyrodextrins but not in the soluble starch. These signals may correspond to the different β -bonds. The coalescence of peaks hampered our attempt to investigate whether starches from various sources behave differently during the pyrodextrinisation process like, for instance, rendering different proportions of β -bonds, as has been suggested from the methylation analysis of non-digestible corn and potato pyrodextrins (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b).

Interestingly, the two unknown signals present in potato pyrodextrin at both 5.1 – 5.0 and 3.5 – 3.4 ppm (Table 4.2) were also present in the other pyrodextrins. As

those signals did not appear in the soluble starch spectra, they are likely to represent structural features due to pyrodextrinisation.

4.3.4. Effect of dialysis on starch pyrodextrins

The *in vitro* fermentation of starch pyrodextrins was performed by using pre-digested samples. At the end of the pre-digestion step, all pyrodextrinised samples were dialysed with a low MW (< 2000) cut-off dialysis bag to remove the digestion products as explained in Chapter 3. This dialysis step should also have washed out low MW pyrodextrins. We were interested, therefore, to study whether there was any change in the structural features found in the pyrodextrins due to the loss of the low MW components. To do so, lentil pyrodextrin was dialysed in the same way as the pre-digested samples and its ^{13}C NMR spectrum was compared with the spectrum of potato pyrodextrin already described in this chapter. Both spectra are shown in Figure 4.5.

All the resonance peaks assigned to anhydro-glucose (Table 4.3), along with four of the signals tentatively assigned to the β bonds, namely $\text{C1}\beta(1\rightarrow2)$, $\text{C2}\beta(1\rightarrow2)$, $\text{C3}\beta(1\rightarrow3)$ and $\text{C4}\beta(1\rightarrow4)$ (Table 4.4), and eight unknown signals disappeared from the spectrum after dialysis. The loss of the anhydro-glucose signals supports the evidence from the ^{13}C NMR spectroscopy that anhydro-glucose, or at least part of it, was not attached to the reducing end of the chain as it has been previously hypothesised (Thompson & Wolfrom, 1958; Theander & Westerlund, 1987) and shown in Figure 4.1.

Table 4.2. ¹H NMR chemical shift values (ppm) for potato pyrodextrin.

	δ range [†] (ppm)	Experimental [‡]	Literature*	
			Ref. A	Ref. B
H1(i), H1(t)	5.4 – 5.3	5.33	5.40 (d)	5.35, 5.33
H4(t)	3.4 – 3.3	3.34 (t, 9)		3.41
H1(a)	5.5 – 5.4	5.39		
H?(a)	4.6 – 4.5	n.r.		
H6(a)	4.1 – 4.0	n.r.		
H1(α -r)	5.2 – 5.1	5.15 (d, 4)	5.27 (d)	
H1(β -r)	4.6 – 4.5	n.r.	4.68 (d)	
H2(β -r)	3.2 – 3.1	—		
H1 α (1→6)	5.0 – 4.8	4.89	5.00 (d)	4.94
H1 β (1→4)	4.5 – 4.3	n.r.		
H1 β (1→6)	4.5 – 4.3	n.r.		
H6 β (1→6)	4.2 – 4.0	n.r.		
Unknown	5.1 – 5.0	5.06 (d, 4)		
Unknown	3.5 – 3.4	3.42 (t, 9)		

(i), Internal residue; (t), non-reducing end or terminal residue; (a), 1,6-anhydro- β -D-glucose residue; (α -r), α -anomer reducing end residue; (β -r), β -anomer reducing end residue; n.r., not resolved signals.

* Ref. A, Gidley, (1985); Ref. B, Nilsson *et al.*, (1996).

[†] Chemical shift ranges were assigned empirically using spectra from several mono-, di- and oligo-saccharides (see section 4.3.1 for further details).

[‡] Multiplicity (d, doublet; t, triplet) and coupling constant (*J*, in Hz) are given between brackets. Spectrum shown in Figure 4.2. Potato pyrodextrin prepared at 10 g/L in D₂O.

Table 4.3. ^{13}C NMR chemical shift values (ppm) for potato pyrodextrin.

	Experimental [†]	Literature*	
		Ref. A	Ref. B
C1(i)	100.8	100.6	
C2(i)	72.7	72.6	
C3(i)	74.5	74.2	
C4(i)	77.9	78.4 or 78.3	
C5(i)	72.3	72.3	
C6(i)	61.6	61.6	
C1(t)	100.9	100.8, 100.7	
C2(t)	72.9	72.8	
C3(t)	74.0	73.9, 74.0	
C4(t)	70.8 – 70.5	70.5	
C5(t)	73.9	73.7	
C6(t)	61.6	61.6	
C1(a)	102.6		102.1
C2(a)	71.4		70.9
C3(a)	73.7		73.3
C4(a)	72.0		71.6
C5(a)	77.4		76.9
C6(a)	66.4		65.8
C1(α -r)	93.1	92.9	
C2(α -r)	—	72.3	
C3(α -r)	74.2	74.1	
C4(α -r)	—	78.6	
C5(α -r)	71.1	71.0	
C6(α -r)	—	61.6	
C1(β -r)	96.9	96.8	
C2(β -r)	75.2	75.0, 75.1	
C3(β -r)	77.0	77.1	
C4(β -r)	—	78.4	
C5(β -r)	75.7	75.6	
C6(β -r)	—	61.8	

(i), Internal residue; (t), non-reducing end or terminal residue; (a), 1,6-anhydro- β -D-glucose residue; (α -r), α -anomer reducing end residue; (β -r), β -anomer reducing end residue.

* Ref. A, Bock *et al.*, (1984); Ref. B, Ritchie *et al.*, (1976).

[†] Spectrum shown in Figure 4.3. Pyrodextrin solution prepared at 100 g/L in D_2O . The chemical shift values were parallel-shifted to give C6(i) a value of 61.6 ppm.

Table 4.4. ^{13}C NMR tentative chemical shift values (ppm) for branching bonds in potato pyrodextrin.

Bond	Atom	Experimental [†]	Ref. A*
Starch branching bond			
$\alpha(1\rightarrow6)$	C1	98.9	98.5
	C6	66.3	66.5
Non-starch bonds			
$\beta(1\rightarrow2)$	C1	105.1	104.4
	C2	81.3	81.4
$\beta(1\rightarrow3)$	C1	103.8	103.2
	C3	80.5	83.5
$\beta(1\rightarrow4)$	C1	104.2	103.6
	C4	79.0	79.9
$\beta(1\rightarrow6)$	C1	103.7	103.0
	C6	69.4	69.4

* Ref. A, Bock *et al.*, (1984).

[†] Spectrum shown in Figure 4.3. Pyrodextrin prepared at 100 g/L in D_2O . The chemical shift values were parallel-shifted to give C6(i) a value of 61.6 ppm.

Table 4.5. ^{13}C NMR chemical shift values (ppm) for lentil pyrodextrin after dialysis.

Lentil pyrodextrin after dialysis*	
C1(i)	100.7
C2(i)	72.6
C3(i)	74.4
C4(i)	77.9
C5(i)	72.3
C6(i)	61.6
C1(t)	100.9
C2(t)	72.8
C3(t)	74.1
C4(t)	70.7 – 70.4
C5(t)	73.9
C6(t)	61.6
C1(a)	Noise
C2(a)	—
C3(a)	—
C4(a)	(72.0)
C5(a)	—
C6(a)	(66.4)
C1(α -r)	93.1
C2(α -r)	—
C3(α -r)	74.3
C4(α -r)	—
C5(α -r)	71.1
C6(α -r)	—
C1(β -r)	97.0
C2(β -r)	75.2
C3(β -r)	77.0
C4(β -r)	—
C5(β -r)	75.7
C6(β -r)	—

(i), Internal residue; (t), non-reducing end or terminal residue; (a), 1,6-anhydro- β -D-glucose; (α -r), α -anomer reducing end residue; (β -r), β -anomer reducing end residue.

Spectrum shown in Figure 4.5. Pyrodextrin solution prepared at 100 g/L in D_2O after dialysis (cut-off 2000) and freeze-drying. The chemical shift values were parallel-shifted to give C6(i) a value of 61.6 ppm.

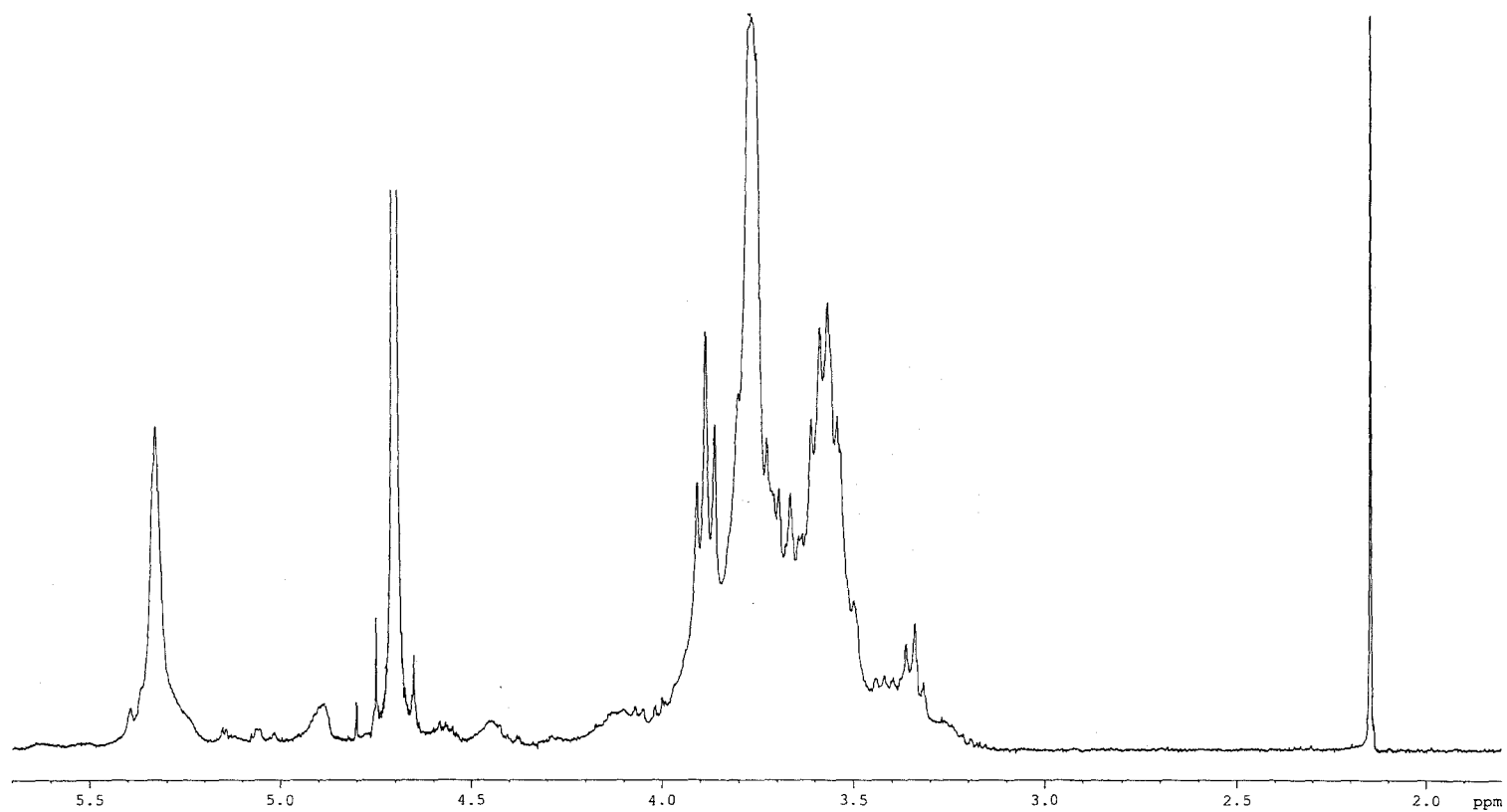


Figure 4.2. ^1H NMR spectrum for potato pyrodextrin.

Potato pyrodextrin solution (10 g/L) was prepared in D_2O . Chemical shift values were parallel-shifted to give HOD a value of 4.70 ppm. Acetone $\delta = 2.147$ ppm.

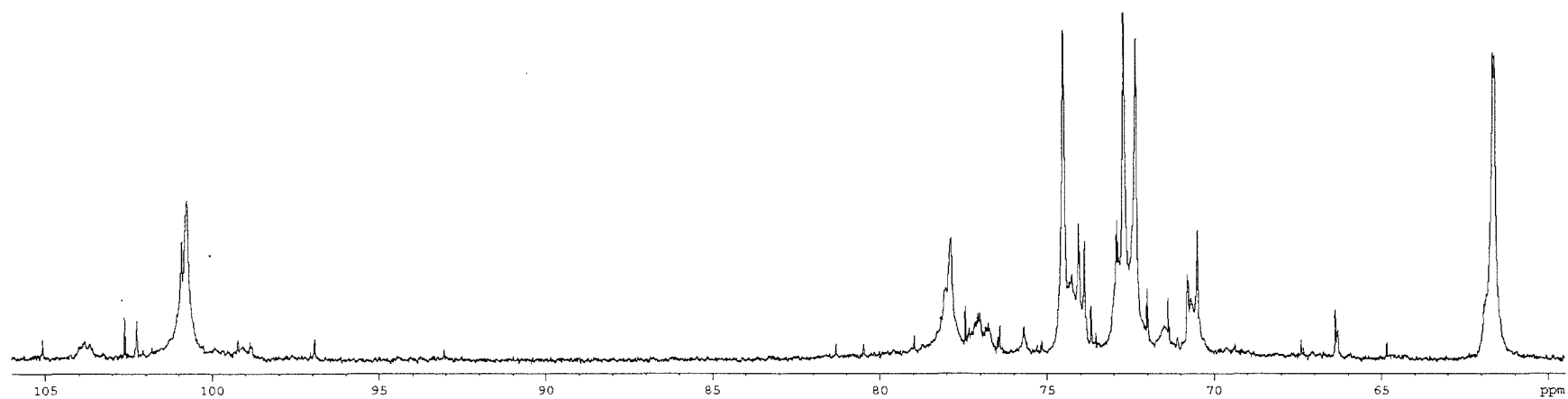


Figure 4.3. ^{13}C NMR spectrum for potato pyrodextrin.

Potato pyrodextrin solution (100 g/L) was prepared in D_2O . Chemical shift values were parallel-shifted to give C6(i) a value of 61.6 ppm.

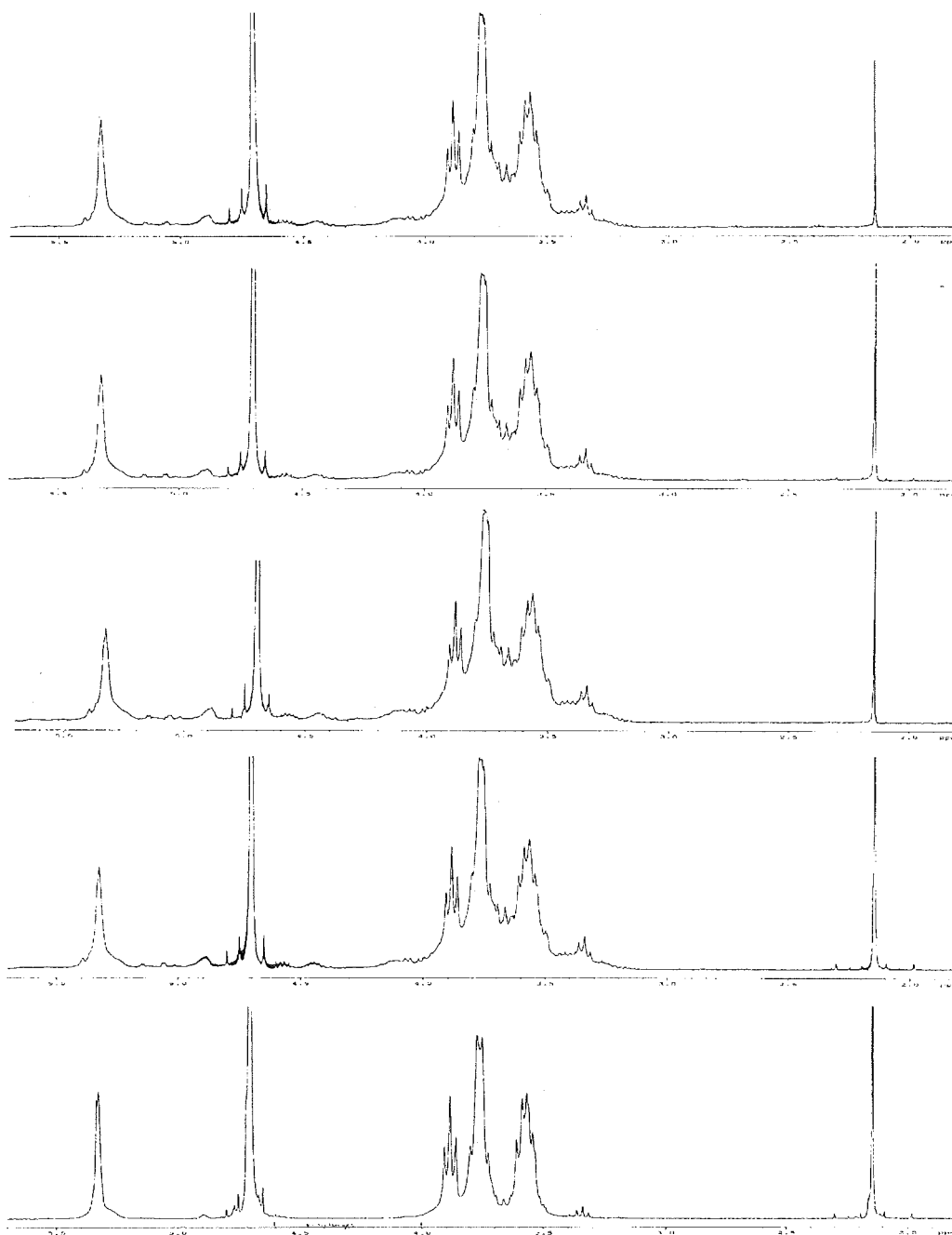


Figure 4.4. Fingerprint ^1H NMR spectra for pyrodextrins and soluble starch. From top to bottom: cocoyam, corn, potato (as shown in Figure 4.2), lentil pyrodextrins and soluble starch. Individual pyrodextrin and soluble starch solutions (10 g/L) were prepared in D_2O . Chemical shift values were parallel-shifted to give HOD a value of 4.70 ppm. Acetone δ was 2.145 (SD 0.001) ppm ($n = 5$).

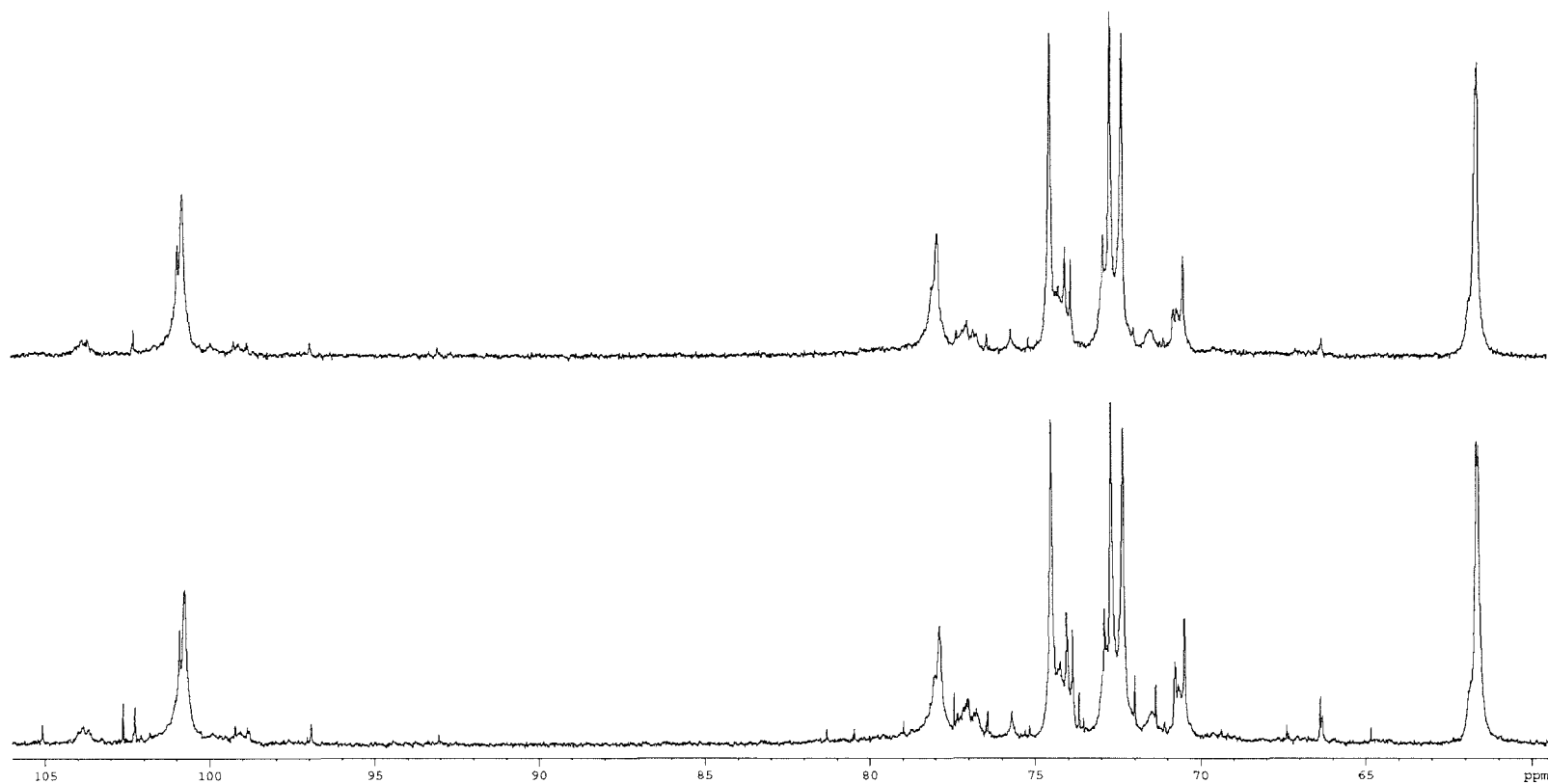


Figure 4.5. Effect of dialysis on starch pyrodextrin preparations.

^{13}C NMR spectra for lentil pyrodextrin after dialysis (top) and potato pyrodextrin before dialysis (bottom, as shown in Figure 4.3). Both solutions were prepared at 100 g/L in D_2O . Chemical shift values were parallel-shifted to give C6(i) a value of 61.6 ppm. 2000 MW cut-off dialysis bag was used.

4.4. Discussion

Elucidation the structure of molecules such as starch pyrodextrins is a complex task not only because carbohydrates are complex molecules by themselves, but also because a variety of different pyrodextrins can be produced by changing the experimental conditions, making more difficult the comparison of results from different studies. However, it was clear from the early work on pyrodextrin structures (Brimhall, 1944; Kerr & Cleveland, 1953; Geerdes *et al.*, 1957) that they were more branched and with lower molecular weight than their native starches.

Using NMR spectroscopy as a tool, we attempted to investigate the chemical structure of a potato pyrodextrin, pyroconverted at 140 °C during 3 h and with 1.82 g acid/kg starch (Ghali *et al.*, 1979). The structure of the main backbone of this pyrodextrin, as shown by the ^{13}C NMR experiment, was similar to that of wheat amylopectin (Dais & Perlin, 1982) and potato starch (Nilsson *et al.*, 1996), suggesting that an important proportion of the potato pyrodextrin were composed of the same $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ bonds of the starch.

Moreover, the presence of glucosidic linkages different from those present in the starch has also been known for sometime (Thompson & Wolfrom, 1958). The most abundant of the non-starch linkages in the pyrodextrin prepared in an acidic environment (as in this thesis) seems to be the $(1\rightarrow3)$ bond. In fact, the $(1\rightarrow3)$ bond accounted for 7 – 14 % and 8 – 11 % of the linkages in potato (Ohkuma *et al.*, 1993a) and corn (Ohkuma *et al.*, 1993b) pyrodextrins, respectively. However, the anomeric conformation of this or any other non-starch bonds present in acid-converted pyrodextrins have not been reported.

In this thesis, we were able to show the presence of non-starch bonds showing the β conformation. In a molecule such as glucose, the constituent of starch polymers, four types of β bonds are possible: $\beta(1\rightarrow2)$, $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$. Even though we found evidence for the presence of some β bonds in both ^1H and ^{13}C NMR spectra of potato pyrodextrin, the overlapping of signals in the ^1H NMR and the low signal-noise ratio in the ^{13}C NMR, along with the lack of more suitable structural models from the literature to compare our data with, hampered our attempt to assign definite chemical shifts to these bonds. However, it seems that the $\beta(1\rightarrow2)$ bond, and not the $\beta(1\rightarrow3)$ bond, was the most abundant in the potato pyrodextrin studied.

An interesting result was found with respect to anhydro-glucose. It has been postulated that this compound is attached to the reducing end of pyrodextrins (Thompson & Wolfrom, 1958; Theander & Westerlund, 1987), but the evidence presented here suggests that the anhydro-glucose is split from the polymer. This behaviour is more likely to occur during starch pyrolysis in vacuum, as has been suggested by Lowary & Richards (1991) and discussed in Chapter 1.

NMR spectroscopy provided useful yet limited information about the structure of potato pyrodextrins. To go further, however, it is necessary to use other experimental approaches. Fragmentation of the pyrodextrin molecule, for instance, either by partial acid hydrolysis or by enzymatic means, could provide the possibility of purifying smaller and therefore less complex molecules than pyrodextrins. This would enable a higher signal to noise ratio and a clearer image of the potential bonds. However, this approach would need extensive fractionation of the pyrodextrin preparation, separation and purification based on molecular

weight and other properties and finally detailed two-dimensional NMR spectroscopy. There would be no guarantee that this would result in sufficiently clear NMR spectra to fully establish the bonds and structure. Indeed, this approach would only be possible for the low molecular weight molecules produced during pyrodextrinisation and would not be possible with the polymer molecules. The main limitation, apart from the time taken to purify the molecules is the time taken for the NMR scan of a polymer molecule. The purification of the small molecules could be carried out using activated charcoal columns, but this would require years of detailed chemistry and was not possible in this PhD.

Chapter 5.

Choice of pyrodextrin for human study: Canary Islands Gofio or Fibersol-2

This chapter describes two commercial products, Canary Islands gofios and Fibersol-2, as possible substrate candidates to test the physiological effect of starch pyrodextrins. This was required, as we could not produce sufficient quantities of our own starch pyrodextrins at food grade for human feeding studies.

5.1. *Introduction*

To test any physiological effects of starch pyrodextrins in humans, it is necessary to scale up the pyrodextrinisation process, which has been used in the laboratory, to produce pyrodextrins on a pilot scale. This laboratory scale to pilot scale step usually involves re-evaluating and readjusting the experimental conditions to optimise the experimental protocols. It also needs a pilot plant where this optimisation can be carried out.

Pyrodextrin production involves four steps (Wurzburg, 1986a): pre-treatment, pre-drying, pyroconverting (or heating) and cooling as discussed in Chapter 1. In the production of pyrodextrins in laboratory scale (Chapter 2), the pre-treatment was done by spraying 22 g of starch with 0.5 mL of a catalyst solution, i.e. 2.2 mol/L hydrochloric acid. To achieve this step in a pilot scale, it would require the use of horizontal or vertical mixers (Evans & Wurzburg, 1967) to blend the starch with the catalyst and guarantee the uniform distribution of the additive.

In the laboratory scale, both the pre-drying and the pyroconversion itself were carried out simultaneously during the 3-hour heating at 140 °C. For larger

amounts of starch, these two steps may well be carried out separately to make sure that the moisture content of the starch is appropriate before the pyroconversion begins. The pre-drying would require, for instance, the use of a stream of hot air. The pyroconversion, on the other hand, could be made in a mixer equipped with a heating system or on belts (with the starch laying as a thin layer over the belts) moving through heated ovens (Evans & Wurzburg, 1967).

Finally, the cooling step was achieved by simply letting the pyrodextrins cool down at room temperature for the laboratory preparation. This is not possible on a pilot scale given the much larger quantities used, therefore, a cooling step would be required to stop the pyroconversion when a desired property (for example, *in vitro* digestibility) is achieved.

In summary, all pyrodextrinisation steps would require optimisation to reach pyrodextrins with at least similar solubility and digestibility *in vitro* as those prepared in laboratory scale. To achieve this, the Glasgow School of Food Technology (Glasgow) was contacted to see whether they had the equipment and qualified personnel to produce the pyrodextrins for our pilot study. Unfortunately, they did not have the appropriate equipment to carry out the pre-drying and pyroconversion steps; we therefore chose to use a commercially available pyrodextrin, which would be different to those in the *in vitro* studies, but of comparable type and structure.

In the following sections, the characteristics of two commercial products, which could act as the pyrodextrin source, are described. Comparison of their digestibility, fermentation properties and structure would allow choice of the most appropriate source for the human experiments.

5.1.1. Gofio

One possible source of pyrodextrins was the gofios. I came across with them reading Isabel Allende's novel "Of love and shadows". She described in her book a soft drink that she called "flour water" and is made from toasted flour:

*"'Get out the mugs for the flour water, daughter,'
Digna directed.*

*Evangelina began to sing as she lined up aluminum
and enameled-tin receptacles on the table. Into each
she measured a couple of tablespoons of toasted
flour and a little honey. Later they would add fresh
water to offer to the visitors who arrived at the hour
of the trance in hopes of being benefited by some
minor miracle."*³

What drew my attention to "flour water" was the fact that it was made from flour that became soluble in water after toasting; resembling the way pyrodextrins are produced by heat treatment with or without the presence of a catalyst (usually, an acidic environment) (Wurzburg, 1986a).

After an anecdotal search, I realised that "flour water" was referring to gofios, a food that has been traditionally produced and consumed by Canary Islands people since pre-Hispanic times, although their consumption has also spread to Northern Africa and South America (Sitjar de Togores *et al.*, 1988). Gofios made in the Canary Islands are protected by a geographical indication as "Canary Islands Gofio" (EEC Council Regulation No. 2081/92, cited by Febles *et al.*, 2000). There are, therefore, Canary Islands wheat-gofio, Canary Islands corn-gofio and so on. In this thesis, the generic term "gofio" is used to refer to gofios produced and consumed not only in Canary Islands, but also in other parts of the world.

³ Allende, I. (1984) Of love and shadows (M. Sayers Peden, translator). Reading: Black Swan. p 43.

Gofios are produced by roasting grains and then grinding them to produce flour. Traditionally, the roasting was carried out in ceramic ovens and the grinding with hand driven stone mills, although in modern times electric mills are used (Mora Morales, 1991). These stone mills are made by two stones chiselled like domes: the circular flat area between the stones is the grinding surface in which roasted grains are added through a hole made in the upper stone. The upper stone also have several holes on its side, to allow its circular movement against a ground fixed lower stone (González, 2002). It is claimed that gofios ground by hand have better flavour and texture (Mora Morales, 1991).

Gofios can be made from different grains. The most common gofios are made from wheat, corn, barley and rye, either individually or in mixtures (Sanchez Reyes, 1903). Although they have been produced using other sources like: unripe bananas, fern roots, chick-peas and beechnuts, among others (Mora Morales, 1991). It is believed that barley-gofio was the only one produced before the Spaniards conquerors arrived in the Canary Islands. Corn-gofio, on the other hand, was produced after the conquerors brought corn from the New World, i.e. America (Mora Morales, 1991). Nowadays wheat-gofio and corn-gofio are the most produced, and to a lesser extent, barley mixed with wheat or corn are used to produced mixed gofios (Anonymous, 2002).

Canary Islands emigrants took the gofio tradition to South America. Today, gofios are produced in Uruguay, Brazil, Chile, Argentina and Venezuela (Anonymous, 2002). In these countries, gofios are known by different names, for example, Venezuelans call “fororo” the flour made with roasted corn grains and “gofio” the one made from wheat grains, whereas Chileans call the latter “ulpo”.

In the Canary Islands, gofios are used to prepare quite a variety of drinks, meals and desserts (Mora Morales, 1991). As drinks, they can be mixed with water, warm milk, coffee, tea or even red wine. As meals, gofio is mixed with soups or cereals, and fried with butter or oil, among other recipes. Custard-like dishes, mousse, pies and cakes are prepared with gofios. Finally, perhaps the most common way to eat gofio is as gofio-dough. This is prepared by kneading the flour with water and/or olive oil to form a dough, adding honey or sugar to taste. Alternatively, the flour is kneaded with water and some pieces of white and soft cheese, bananas, cooked potatoes and/or cooked sweet potatoes, milk, cane syrup or butter (Mora Morales, 1991; Anonymous, 2002). In the Canary Islands, anecdotal information suggests a gofio intake of 10 g/d.

From a scientific viewpoint, research on gofios has considered the phytic acid levels and their effect on the intestinal absorption of zinc. Febles *et al.* (2000) screened almost 400 samples of gofios (90 wheat-gofios, 120 corn-gofios, 60 rye-gofios, 110 barley-gofio) taking into account their production, sales volume and location in the island of Tenerife (Spain). They used indirect iron (III) complexometry with sulfosalicylic acid as titration end-point indicator and found that gofio samples had 7.0 g/kg phytic acid on average, with most of the samples having phytic acid levels between 3 to 12 g/kg. The corn-gofio phytic acid content was significantly lower than those for rye- and wheat-gofio, but similar to barley-gofio (Febles *et al.*, 2000).

Sitjar de Togores *et al.* (1988) studied the effect of wheat-gofio on the intestinal absorption of zinc in the rat. They fed rats consecutively with three diets (26 days per each diet): the rat habitual diet (with a mixture of wheat, oats, corn and barley

flours), a wheat flour diet (like habitual diet, but with wheat flour only) and a wheat-gofio diet (like the habitual diet, but with wheat-gofio flour only). They found a significant increase (58 %) in faecal zinc concentration and a significant decrease (-66 %) in zinc levels in hair, at the end of the wheat-gofio diet compared with the end of the habitual diet. The authors argued that wheat-gofio could compromise the zinc balance in rat, decreasing the intestinal absorption of this ion, possible by interactions with phytic acid and other gofio components, like dietary fibre (Sitjar de Tógores *et al.*, 1988). However, it is important to note that the gofio intake used by those authors was very high (77 % of the diet). There have been no studies of the digestibility and fermentability of gofios.

5.1.2. Fibersol-2

An alternative source of commercial pyrodextrin is Fibersol-2. Fibersol-2 is the registered name for a commercial non-digestible corn pyrodextrin developed and commercialised in Japan by Matsutani Chemical Industry Co. Ltd. Similar to the pyrodextrins studied in Chapter 3, Fibersol-2 is produced by a combination of heat and hydrochloric acid in a low-moisture environment. The actual pyroconversion conditions are an industrial secret, but it is likely that Fibersol-2 is produced at 150 – 180 °C, 15 – 60 min and 0.75 – 1.20 g HCl/kg starch (Ohkuma *et al.*, 1993b). Unlike the pyrodextrins studied in Chapter 3, Fibersol-2 is submitted to an enzymatic treatment with α -amylase and glucoamylase that hydrolyses most of the digestible components and then is deionised, decolourised, purified and spray-dried (Ohkuma *et al.*, 1990; Ohkuma & Wakabayashi, 2001). The result is a white powder, water-soluble saccharide with low viscosity, low molecular weight (about 2000), a dextrose equivalent less than 20 and with non-starch bonds,

particularly (1→3) bonds (Ohkuma *et al.*, 1993b). Fibersol-2 is recognised as an “indigestible dextrin” type in Japan (Ohkuma & Wakabayashi, 2001).

Fibersol-2 and other non-digestible pyrodextrins seem to be safe. Wakabayashi *et al.* (1992a) reported no mortality in male mice over a 7 day period when they were fed with a single dose of up to 20 g/kg of Pine Fibre-C⁴. Using another commercial product, Pine Fibre⁵, Satouchi *et al.* (1993) estimated that a single dose of 2.4 g/kg body weight was needed to produce diarrhoea in half of 74 healthy adults. Several long-term studies (12 weeks) with Pine Fibre-C (Mizushima *et al.*, 2000) or Fibersol-2 (Kishimoto *et al.*, 2000b; Shioda *et al.*, 2001; Kajimoto *et al.*, 2001; Sekizati & Yonezawa, 2001) intake from 18 to 30 g/d have shown no significant changes in blood laboratory tests, such as haematological counts, electrolyte concentration and markers for protein metabolism, renal, pancreatic and hepatic functions, as well as urine analysis, blood pressure and physical examination. Fibersol-2 also meets the American “generally recognised as safe” (GRAS) requirements as set forth in 21 CFR 184.1444 (Maltodextrins, 1996; as cited by Tsuji & Gordon, 1998).

There are several products commercialised in the Japanese market that contain Fibersol-2 as an added ingredient: “Iikoto tea” (green tea based drink) (Kajimoto *et al.*, 2001), “Kenji-Salon” (canned tea drink) (Tokunaga & Matsuoka, 1999), “Supli” (grapefruit flavoured drink) (Umekawa *et al.*, 1999), “Kaiteki Senn-i prune jelly” (Kimura *et al.*, 1998), cookies (Ogiso *et al.*, 1999), “Nata de Coco”

⁴ Pine Fibre-C is the registered name for a non-digestible pyrodextrin similar to Fibersol-2, but made from potato starch by Matsutani Chemical Industry Co. Ltd. (Itami city, Japan).

⁵ Pine Fibre is another product of Matsutani Chemical Industry Co. Ltd (Itami city, Japan) for the Japanese market. It contains 65 % Pine Fibre-C (non-digestible potato pyrodextrin) and 35 % maltodextrin. Pine Fibre has an average MW of 1600 and contains 58 % fibre (Satouchi *et al.*, 1993).

(traditional food made from fermented coconut milk) flavoured yoghurt (Unno *et al.*, 2000), “Dr Drink Yoghurt” (Shioda *et al.*, 2001), “Sukoyaka tofu” (healthy tofu) (Uno *et al.*, 1999), “Midori No Kenko Yasai” (vegetables and fruits mix juice) (Tanaka *et al.*, 2000), “Dietary Fibre and Peach” (peach juice) (Yamamoto *et al.*, 2000) and “Matsutani-no Omisoshiru” (Matsutani’s Miso soup; awase, akadashi and shiromiso flavoured) (Kishimoto *et al.*, 2000b). Some of these products have the “Food for Specified Health Use” (FOSHU) status in Japan and they are used, for instance, “(to) help maintain the intestinal regularity” (Yamamoto *et al.*, 2000) and “to improve diet of people concerned about their blood glucose levels” (Tokunaga & Matsuoka, 1999). Fibersol-2 is also commercialised in North America as the dietary fibre source for the Campbell’s Center for Nutrition and Wellness (Campbell Soup Co., Camden, NJ, USA) plan, which is a complete nutritional plan that appears to produce favourable changes in patients with high risk of cardiovascular disease (McCarron *et al.*, 1997).

There is no published data of the effect of Fibersol-2 on intestinal bacteria.

Ohkuma *et al.* (1990) showed that some species of Bacteroides and one species of Bifidobacteria were able to use Pine Fibre-C as a substrate. However, Satouchi *et al.* (1996) found that a non-digestible depolymerised pyrodextrin, prepared from a corn pyrodextrin (pyroconversion at 145 °C for 30 min with 0.67 g HCl/kg starch) by partial acid hydrolysis (125 °C for 20 min) and pre-digestion with glucoamylase, was fermented by several species of Bifidobacteria, Lactobacilli and Bacteroides.

The fermentation properties of Fibersol-2 have been studied using rat caecal contents as an inoculum for static cultures. A molar ratio of 38:19:43 for acetate,

propionate and butyrate, respectively, was reached after 24 h of anaerobic incubation (Kishimoto *et al.*, 1995). More recent, Flickinger *et al.* (2000) reported a molar ratio of 70:21:9 for acetate, propionate and butyrate, respectively, also after 24 h of anaerobic incubation but using human faeces as inoculum for static cultures. This would suggest that Fibersol-2 has the high proportion of propionate we would require for our studies, but potentially a different proportion of acetate and butyrate.

5.1.3. Aim

The aim of this work was to test the suitability of Canary Islands gofios and Fibersol-2, two commercially available products, for their use as substrate for human trials to test the possible physiological effects of pyrodextrins.

5.2. Materials and Methods

5.2.1. Samples

Both wheat-gofio and corn-gofio were supplied by Molinería Miraflor (Las Palmas de Gran Canaria, Spain). Fibersol-2 was supplied by Matsutani Chemical Industry Co. Ltd. (Itami, Japan).

5.2.2. Criteria used

Two criteria were used to test the suitability of the commercial pyrodextrins as a substrate for human trials. First, the resistant carbohydrate content was measured. It had to be high enough to allow the incorporation of the substrate into the subject diets without drastically altering their eating habits. Second, the SCFA profile after 24 hour *in vitro* fermentation was assessed. The SCFA profiles had to be as similar as possible to those obtained for the pyrodextrin already studied in this thesis (see Chapter 3).

5.2.3. Determination of starch fractions

Rapidly digestible, slowly digestible, resistant and total starch *in vitro* for wheat-gofio, corn-gofio and raw potato starch (for comparison purposes) were estimated using Englyst Starch kit (Englyst Carbohydrate Services Ltd., Cambridge, UK) (Englyst *et al.*, 1992). In this method, starch is hydrolysed by different enzymes and the glucose released is quantified at 20 (rapidly digested) and 120 min (slowly digested). Finally, the remaining starch (resistant starch) is dispersed and enzymatically hydrolysed once again to obtain total glucose. The experimental protocol is summarised in Figure 5.1. The different starch fractions were calculated according to Englyst *et al.* (1992) using the following formulae:

$$\begin{aligned}TS &= 0.9 \times (TG - FG) \\RDS &= 0.9 \times (G20 - FG) \\SDS &= 0.9 \times (G120 - G20) \\RS &= 0.9 \times (TG - G120) \\RAG &= G20\end{aligned}$$

Where:

FG is the free glucose

G120 is the glucose released after 120 minutes

G20 is the glucose released after 20 minutes

RAG is the rapidly available glucose in g/100 g

RDS is the rapidly digestible starch in g/100 g

RS is the resistant starch in g/100 g

SDS is the slowly digestible starch in g/100 g

TG is the total glucose

TS is the total starch in g/100 g

5.2.4. Fermentation experiment

Fibersol-2 (n = 4) was fermented using the method developed by Edwards *et al.* (1996) as described in Chapter 2. As Fibersol-2 is made from corn starch, native corn starch (n = 3; pre-digested as described in Chapter 3) was also fermented for comparison purposes. SCFA and pH were analysed as described in Chapter 2.

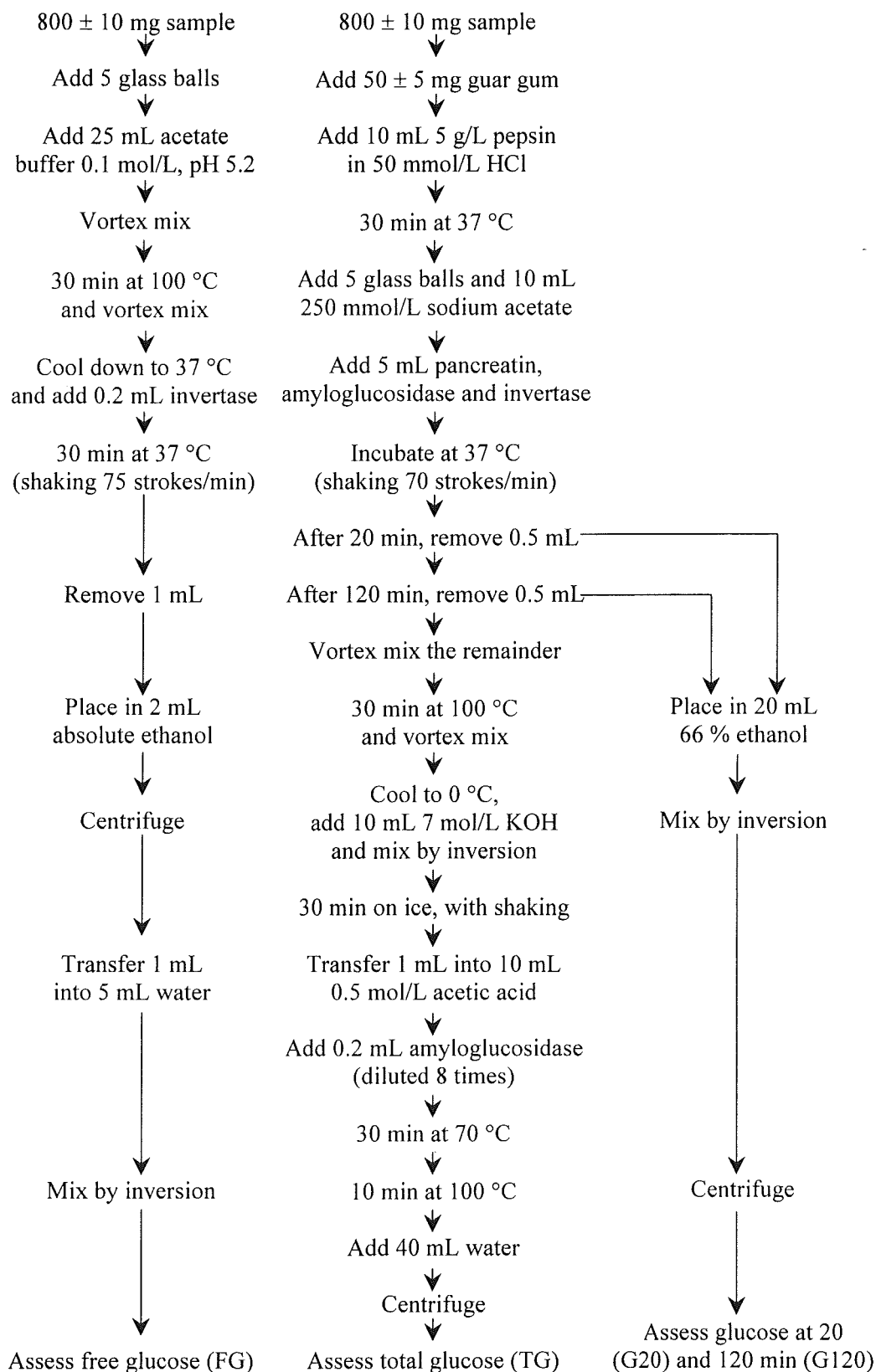


Figure 5.1. Starch fraction protocols.

A summary of the protocols to measure the starch fractions is outlined (Englyst *et al.*, 1992). The formulae to calculate the starch fractions are given in the text. Glucose assay is described in Chapter 2.

5.2.5. NMR spectroscopy

¹H NMR spectroscopy was used to compare the structural features of Fibersol-2 with those of potato pyrodextrin. The spectrum was obtained using the same conditions described in Chapter 4.

5.2.6. Bifidogenic effect

As an added experiment, the chosen substrate was tested to see whether it could selectively increase the growth of Bifidobacteria and Lactobacilli (i.e. act as a prebiotic). To do so, a set of culture McCartney bottles with and without 100 mg Fibersol-2 were fermented *in vitro* for 24 h as described in Chapter 2. Then, the cultures were serially diluted and each dilution was plated on selective media for bacterial quantification as described below. The faeces were donated by six different subjects (2 female and 4 male subjects; age 34 (range 30, 46) years old). Mr Alexander Fletcher (Division of Developmental Medicine, University of Glasgow) kindly prepared the faecal slurries for this experiment.

5.2.7. Bacteria quantification – quantitative method

The quantitative analysis of the colony forming units was done by the modified Miles Misra plate counting technique (Hoben & Somasegaran, 1982). Culture samples were serially diluted down to 1×10^{-8} using sterile phosphate buffered saline (PBS, pH 7.3) solution (100 μ L of sample in 900 μ L of PBS). Then, 20 μ L aliquots from each dilution were drop-plated in triplicate on pre-dried (pre-drying for 25 – 35 min at 45 – 55 °C) Bercs, Rogosa and Columbia blood agar (CBA) plates (preparation details are given in Chapter 2) to grow Bifidobacteria, Lactobacilli and general anaerobes, respectively. The plates were divided into quarters to allow plating of four different dilutions per Petri dish. Plates were incubated upright overnight, inverted on the following morning and re-incubated

at 37 °C in anaerobic conditions using anaerobic jars for a total of 72 h. Colonies were counted in drops which contained between 20 to 60 isolated colonies. The concentration of colony forming units (CFU) was calculated and expressed as CFU/mL. Dr William Mackay (Division of Developmental Medicine, University of Glasgow) helped me by doing both dilutions and drop-plating from some subject samples.

PBS was prepared as follows: 8.30 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogen phosphate and 0.2 g potassium dihydrogen phosphate were dissolved in 1 L water. Before use, 0.1 mL of 5 % L-cysteine solution was added to 9.9 mL PBS as a reducing agent.

5.2.8. Statistical analysis

Variables were described as mean \pm standard deviation. Both the starch fractions of gofios and the fermentation profile of Fibersol-2 were compared using unpaired t-test. The bifidogenic effect of Fibersol-2 was assessed using the Wilcoxon signed rank test.

5.3. Results

5.3.1. Gofio

Table 5.1 shows the different dietary starch fractions for the two gofios and raw potato starch for comparison purposes. Total starch was 59.9 (SD 1.1) and 65.2 (SD 0.6) g/100 g for wheat- and corn-gofio ($n = 4$; $p = 0.001$, unpaired t-test), respectively. In both samples, almost 80 % of the starch was rapidly digestible, but corn-gofio had twice the amount of slowly digestible starch. Resistant starch content was low (< 5 g/100 g) and similar ($p = 0.3$) for both gofio samples.

Table 5.1. Dietary starch fractions for gofio samples.

Sample	Starch fractions (g/100 g wet basis)				RAG
	RDS	SDS	RS	TS	
Corn-gofio	47.3 ± 1.1 ^a	13.4 ± 2.3 ^a	4.5 ± 1.7 ^a	65.2 ± 0.6 ^a	52.6 ± 1.3 ^a
Wheat-gofio	50.4 ± 1.3 ^b	6.4 ± 1.2 ^b	3.2 ± 1.3 ^a	59.9 ± 1.1 ^b	56.1 ± 1.4 ^b
Raw potato starch	4.3 ± 0.9 ^c	22.1 ± 0.6 ^c	46.8 ± 1.8 ^b	73.2 ± 0.6 ^c	4.9 ± 1.0 ^c

Values are mean ± standard deviation (n = 4).

In each column, means with different superscripts were significantly different ($p < 0.05$; - unpaired t-test).

RAG, rapidly available glucose; RDS, Rapidly digestible starch; RS, resistant starch; SDS, slowly digestible starch; TS, total starch.

5.3.2. Fibersol-2

5.3.2.1. Fermentation

During the production of Fibersol-2, this pyrodextrin is hydrolysed with α -amylase and glucoamylase (Ohkuma *et al.*, 1990), eliminating much of the digestible components. In fact, using a consecutive hydrolysis with salivary α -amylase, gastric juice preparation, pancreatic α -amylase and intestinal mucosal enzymes, Tsuji & Gordon (1998) reported a recovery of 89.8 % of the starting material. Thus, we did not consider it essential to evaluate the digestibility of the Fibersol-2 samples and proceed to study its fermentation properties.

Table 5.2 shows net total SCFA, individual SCFA proportions and fall in pH in cultures after 24 h *in vitro* fermentation with human faeces. Fibersol-2 produced a similar profile of individual SCFA to the pyrodextrins studied in Chapter 3, except that the fall in pH that was higher for the commercial pyrodextrin than for the pyrodextrins prepared according to Ghali *et al.* (1979). However, when compared to native corn starch, Fibersol-2 had a significantly higher (20 %) net total SCFA and a significantly lower (26 %) butyrate proportion. The fall in pH with Fibersol-2 was also higher than for corn starch.

Table 5.2. Short-chain fatty acid and fall in pH in fermented cultures of pre-digested native corn starch and Fibersol-2[†].

	Native corn starch [‡] (n = 3)	Fibersol-2 (n = 4)	Potato pyrodextrin ^{‡,§} (n = 4)
Net total SCFA (mmol/L)	47.2 ± 1.0	56.6 ± 2.8**	61.0 ± 5.8
Individual SCFA proportion (%)			
Acetate	56.6 ± 4.0	54.5 ± 1.9	51.1 ± 3.5
Propionate	19.2 ± 5.8	26.6 ± 1.6	25.4 ± 5.9
Butyrate	16.7 ± 1.6	12.4 ± 1.3*	15.1 ± 5.1
iC4–C8	7.5 ± 3.4	6.7 ± 0.8	8.5 ± 4.1
Fall in pH	1.1 ± 0.1	1.7 ± 0.2*	1.0 ± 0.1

Values are mean ± standard deviation.

SCFA, short-chain fatty acids; iC4–C8, sum of iso-butyrate, iso-valerate, valerate, hexanoate, heptanoate and octanoate.

* $p < 0.05$, ** $p < 0.01$ compared with native corn starch (two-sided, unpaired t-test).

[†] Fermentation was carried out in anaerobic incubations with human faeces for 24 h.

[‡] Pre-digested as described in Chapter 3.

[§] Values taken from Chapter 3 for comparison purpose.

^{||} Net total SCFA was calculated by subtracting total SCFA values in control cultures (no carbohydrate) from total SCFA values in test cultures.

5.3.2.2. NMR spectroscopy

The ¹H NMR spectrum for Fibersol-2 is shown in Figure 5.2 and the identified

structural features are summarised in Table 5.3. In general, the Fibersol-2

spectrum resembled that for potato pyrodextrin. However, Fibersol-2 did show

two triplets close together, not present in the pyrodextrin, one at 3.19 ppm (not

assigned) and the other at 3.16 ppm, assigned to H2(β-r). Additionally, the

resonance signals for H1(α-r), H1(β-r) and H1α(1→6), along with those at 4.5 –

4.0 ppm (probably due to the β bonds as explained in Chapter 4) were more

prominent in Fibersol-2 when compared to those in potato pyrodextrin.

Table 5.3. ¹H NMR chemical shift values (ppm) for Fibersol-2.

	δ range* (ppm)	Experimental [†]
H1(i), H1(t)	5.4 – 5.3	5.32
H4(t)	3.4 – 3.3	3.34 (t, 8)
H1(a)	5.5 – 5.4	5.39
H?(a)	4.6 – 4.5	n.r.
H6(a)	4.1 – 4.0	n.r.
H1(α -r)	5.2 – 5.1	5.15 (d, 4)
H1(β -r)	4.6 – 4.5	n.r.
H2(β -r)	3.2 – 3.1	3.16 (t, 8)
H1 α (1→6)	5.0 – 4.8	4.89 (d, 4)
H1 β (1→4)	4.5 – 4.3	n.r.
H1 β (1→6)	4.5 – 4.3	n.r.
H6 β (1→6)	4.2 – 4.0	n.r.
Unknown	5.1 – 5.0	5.06 (d, 4)
Unknown	3.2 – 3.1	3.19 (t, 8)
Unknown	3.5 – 3.4	3.42 (t, 8)

(i), Internal residue; (t), non-reducing end or terminal residue; (a), 1,6-anhydro- β -D-glucose residue; (α -r), α -anomer reducing end residue; (β -r), β -anomer reducing end residue; n.r., not resolved signals.

* Chemical shift ranges were assigned empirically using spectra from several mono-, di- and oligo-saccharides (see Chapter 4 for further details).

[†] Multiplicity (d, doublet; t, triplet) and coupling constant (J , in Hz) are given between brackets. Spectrum shown in Figure 5.2. Fibersol-2 prepared at 10 g/L in D₂O.

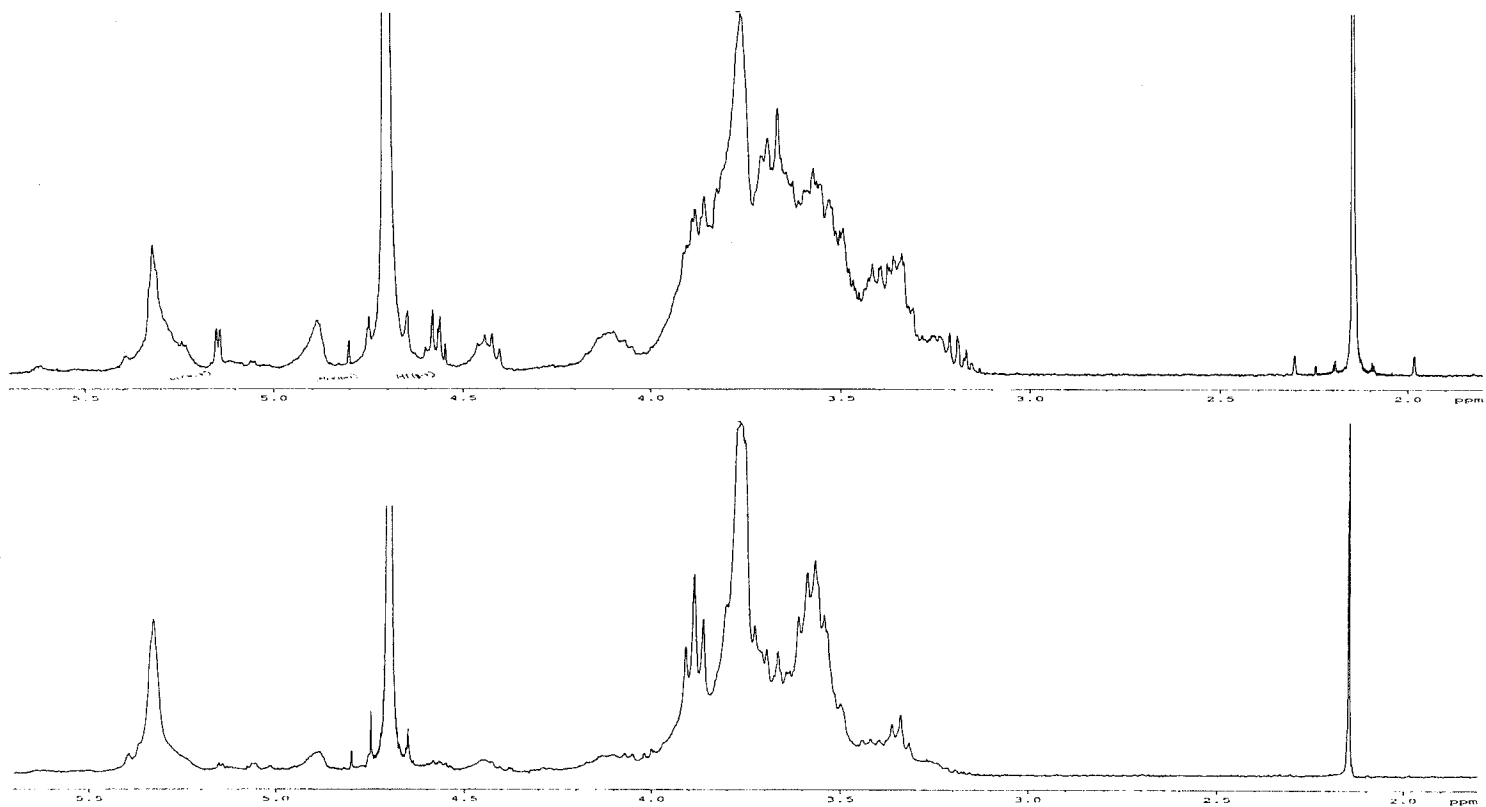


Figure 5.2. Comparison between Fibersol-2 and potato pyrodextrin using NMR spectroscopy.

¹H NMR spectra for Fibersol-2 (top) and potato pyrodextrin (bottom, as shown in Figure 4.2). Both solutions were prepared at 10 g/L in D₂O. Chemical shift values were parallel-shifted to give HOD a value of 4.70 ppm. Acetone δ = 2.145 ppm.

5.3.2.3. Bifidogenic effect *in vitro*

Total anaerobes and Bifidobacteria decreased slightly in the cultures with Fibersol-2 (Table 5.4). Interestingly, Lactobacilli decreased by two orders of magnitude compared with the culture without Fibersol-2. Table 5.5 shows the proportions of the total anaerobes accounted for by Bifidobacteria and Lactobacilli. There was no bifidogenic effect due to Fibersol-2. Moreover, the proportion of Lactobacilli was low in control cultures and almost zero in Fibersol-2 cultures.

Table 5.4. Bacteria counts after 24 hour *in vitro* fermentation with and without Fibersol-2.

	Number of bacteria (CFU/mL)			
	Control		Fibersol-2	
Total anaerobes	1.6x10 ⁹	(2.6x10 ⁸ , 2.9x10 ⁹)	1x10 ⁹	(2.2x10 ⁸ , 3x10 ⁹)
Bidifobacteria	2x10 ⁸	(3.7x10 ⁷ , 4.1x10 ⁸)	1.4x10 ⁸	(5.4x10 ⁷ , 2.9x10 ⁸)
Lactobacilli	2x10 ⁷	(3.3x10 ⁶ , 2.2x10 ⁸)	2x10 ⁵	(4x10 ⁴ , 2.1x10 ⁸) [†]

Values are median (range) n = 6.

[†] p = 0.059 compared with cultures without Fibersol-2 (Wilcoxon signed rank test)

Table 5.5. Proportion of Bifidobacteria and Lactobacilli with respect to total anaerobes after 24 hour *in vitro* fermentation with and without Fibersol-2.

	Proportion of total anaerobes (%)			
	Control		Fibersol-2	
Bidifobacteria	10.4	(4.3, 22.8)	10.6	(5.7, 40.9)
Lactobacilli	1.59	(0.28, 7.59)	0.02	(0.01, 7.00)

Values are median (range) n = 6.

[†] p = 0.059 compared with cultures without Fibersol-2 (Wilcoxon signed rank test)

5.4. Discussion

5.4.1. Gofio

Even though there are several ways to consume gofios (Mora Morales, 1991; Anonymous, 2002), anecdotal information suggests that Canary Islanders consumed them raw, mainly as gofio-dough. We therefore looked into the different starch fractions defined by Englyst for raw flours to see whether gofios met our first criteria of choosing a substrate (high resistant starch content).

Resistant starch for both gofios (Table 5.1) was comparable to breakfast cereals (corn flakes, rice krispies) and fried potato (2.5–5.0 g/100 g) and it was higher than the resistant starch content for wheat and corn flours (< 1.0 g/100 g) (Goñi *et al.*, 1996). However, the resistant starch content was too low in both gofio samples to allow their use as the pyrodextrin for a human trial. If we wanted to feed the subjects with 30 g/d resistant starch, we would have needed 667 g of corn-gofio or 938 g of wheat-gofio per day per person. These amounts are impossible to incorporate into a diet.

There are some differences in the way gofios and starch pyrodextrins are produced. Starch pyrodextrins were made by heat-acid treatment of isolated starches (Chapter 3). Gofios, on the other hand, are made by roasting whole grains and then grinding them to flour (Mora Morales, 1991). Gofios, therefore, are pyroconverted in the presence of all grain components (not only starch, but also protein, fat, dietary fibre, etc.). Additionally, gofio preparation does not involve any catalyst added to the process.

It seems that the presence of the acid catalyst is important to promote the production of non-digestible fractions. Laurentin *et al.* (2003) compared the effect

of the acid catalyst on the available starch content of a lentil pyrodextrin prepared by incubating the starch at 140 °C for 3 h (as in this thesis). They found that with no hydrochloric acid added, the digestible starch level did not change when compared with the native starch, even when the heating time was increased by 3-fold (i.e. 9 hours).

The temperature used to produce the gofios used in this thesis ranged between 165 °C and 175 °C (personal communication from Mr Juan González, Molinería Miraflor, Las Palmas de Gran Canaria, Spain). The transglucosidation reactions that are likely to be the responsible for the non-digestible fractions shown by heat-acid-produced pyrodextrins, may be impaired by the absence of the catalyst when the temperature is below 200 °C (Kroh *et al.*, 1996); explaining the low non-digestible fractions found in gofios.

The amount of slowly digestible starch for corn-gofio was comparable to those of macaroni pasta, kidney beans and oat bran cereal (Englyst & Hudson, 2000). Slowly digestible starch sources may have several physiological benefits, for instance, lower glycaemic responses. As this starch fraction is slowly yet completely digested in the small intestine (Englyst *et al.*, 1992), gofios may provide less extreme postprandial response.

In conclusion, the non-digestible fraction of gofios was too low to suit the requirements of the human trial therefore this option was discarded. However, the starch fractions showed that gofios might provide a significant contribution to slowly digestible and resistant starch in the diet of gofio consumers.

5.4.2. Fibersol-2

Fibersol-2 is a non-digestible corn pyrodextrin with non-starch bonds (Ohkuma *et al.*, 1993b; Ohkuma & Wakabayashi, 2001), most likely in β conformation as shown for potato pyrodextrin in Chapter 4. As the use of highly-specific enzymatic assays such as the Englyst method can underestimate the starch fraction content of Fibersol-2, its use was avoided to check the first selection step. However, it has been reported that Fibersol-2 has a non-digestible fraction of 90 % (Tsuji & Gordon, 1998). We therefore considered this value high enough to meet our first criterion.

The SCFA profile of Fibersol-2 (Table 5.2) was similar to those of the starch pyrodextrins studied in Chapter 3, especially the high propionate ratio.

Consequently, this commercial pyrodextrin met the second criteria. Interestingly, the fermentation properties of native corn starch differed from those properties showed by the other native starches (Table 3.1). Corn starch had a higher propionate to acetate ratio than potato, lentil and cocoyam starches. Moreover, the fall in pH was less marked. There is no explanation for this differential behaviour of corn starch. More research is needed.

As Fibersol-2 met the proposed conditions for the substrate in human studies and taking advantage of the expertise gained with the NMR analyses described in Chapter 4, I was interested to get some insight into the structure of Fibersol-2. Comparing the ^1H NMR spectra of Fibersol-2 with potato pyrodextrin (Figure 5.2), we demonstrated the probable presence of β linkages, although the coalescence of signals hampered attempts to identify fully the type of β -bonds involved. Judging by the height of the peak, however, it seems that Fibersol-2 was

more branched and with higher proportions of β bonds than the starch pyrodextrins produced under the conditions described by Ghali *et al.* (1979).

Finally, Fibersol-2 was tested for any bifidogenic effect *in vitro*. The data showed (Table 5.4) that *in vitro* cultures were not useful for assessing the growth of any particular group of bacteria. One of the main limitations of this system is that bacteria have to go through a lag phase before they actually begin to grow and most of them may not survive this phase. This may have been the case for Lactobacilli. Another limitation of the static cultures is that they are closed systems. The changes in the culture environment due to the increase in products, the decrease in substrates and changes in pH, may differentially affect groups of bacteria. It is important to note that the control culture may have not been the best one for comparing bifidogenic effects, since the absence of a source of carbohydrate may have lead to a rapid death of bacteria. A more suitable control could have been raw corn starch because Fibersol-2 is a non-digestible corn pyrodextrin. However, it is known that corn starch is bifidogenic (Topping *et al.*, 2003). Another option could have been guar gum, a carbohydrate that is fermentable, but not bifidogenic.

In conclusion, Fibersol-2 was more suitable as a substrate than Canary Islands gofios for testing any physiological effect of pyrodextrins in humans.

Chapter 6.

Effect of Fibersol-2 on faecal short-chain fatty acids and bacteria in healthy humans — a doubled blind, pseudo-randomised pilot trial

The study of pyrodextrin fermentation (Chapter 3) carried out with an *in vitro* model of the large intestine showed that pyrodextrins were fermented differently to their native starches. The ratio of propionate to total SCFA produced doubled for the pyrodextrins. We therefore decided to investigate whether this effect was seen *in vivo*. As we were not able to produce starch pyrodextrins on a pilot scale, a commercial non-digestible pyrodextrin —Fibersol-2— was chosen (Chapter 5). For the last year of my PhD, therefore, I designed two human trials in an attempt to investigate the effect of Fibersol-2 on human physiology. The first attempt (Trial 1) was designed to investigate the effect of this non-digestible pyrodextrin on plasma triglyceride levels in men and whether an increase in propionate to acetate ratio during colonic fermentation was responsible for any hypolipidaemic effect (see Appendix 3). Due to problems with recruitment, only two subjects have completed Trial 1. It was clear that Trial 1 would not be completed in the PhD timeframe; therefore, we decided to run another study. The second trial (Trial 2) was designed as a pilot study to investigate the effect of Fibersol-2 intake for one week on SCFA, pH and bacteria in human faeces. The results of Trial 2 are presented in this chapter.

6.1. Introduction

Fibersol-2 is a commercial non-digestible corn pyrodextrin developed in Japan. Few studies on the effect of Fibersol-2 on lipids in the fasting state have been published (Table 6.1). A comprehensive review of these investigations was hampered because they were all printed in Japanese. Matsutani kindly provided translated summaries, but it was not possible to check the data in the original papers. However, it can be concluded that an intake of 29.4 (range 11.7 – 60.0) g/d of Fibersol-2 for four weeks decreased fasting blood triglycerides by 27 (range 19 – 45) % from their baseline levels, 2.32 (range 2.00 – 3.22) mmol/L (n = 7). This effect was more marked in those studies where the sample population had higher baseline blood triglyceride levels (Matsuoka *et al.*, 1992; Nomura *et al.*, 1992; Fujiwara & Matsuoka, 1993; Tokunaga & Matsuoka, 1999; Mizushima *et al.*, 2000; Kajimoto *et al.*, 2000; Kishimoto *et al.*, 2000b).

The fermentation properties of Fibersol-2 have been studied in rats. A decrease in rat caecal content pH and an increase in butyrate was reported when rats were fed a non-digestible potato or corn pyrodextrin enriched diet in comparison with a corn fibre enriched diet (Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b; Kishimoto *et al.*, 1995). Using rat caecal contents as inoculum for static cultures, a molar ratio of 38:19:43 for acetate, propionate and butyrate, respectively, was reached after 24 h of anaerobic incubation (Kishimoto *et al.*, 1995). More recently, Flickinger *et al.* (2000) reported a molar ratio of 70:21:9 for acetate, propionate and butyrate, respectively, also after 24 h of anaerobic incubation, but using human faeces as inoculum for static cultures.

Table 6.1. Summary of published works about the effect of commercial non-digestible pyrodextrins on serum lipids in humans.

Ref. [†]	Subjects	Sample size	Intervention period	Route of administration	Daily intake	Main results in mmol/L [‡] (baseline, at 4 th week, end point)			
D-10	Healthy, male, 33 – 59 y	10	4 week, next 4 week with half dose	10 g powder in 100 mL water with each meal	30 g/d PF-C	TC	5.85	5.55	5.15
						HDLc	1.20	1.15	1.25
						TAG	2.74	2.22	2.00
C-13 (D-7)	Healthy, male	10	12 week	Instant miso-soup, 1 sachet (14 g) per meal	11.7 g/d PF-C	TC	5.85	5.20	5.55
						HDLc	1.35	1.25	1.40
						TAG	2.00	1.46*	1.64
C-16	Healthy	11	12 week	Dr. Drink Yogurt, 1 bottle (180 mL) per meal	23.9 g/d FS-2	No data were shown			
D-6	Healthy	9	12 week	Dietea (canned tea drink), 1 can (190 g) per meal	15.3 g/d FS-2	TC had no change			
						HDLc (no data were shown)			
						TAG	1.36	–	1.10*
D-4	Hyperlipidemic with NIDDM	5	12 week	20 g NDP with each meal	60 g/d NDP	TC	6.85	5.40*	5.40**
						HDLc	1.25	1.20	1.00**
						TAG	2.74	1.52*	2.00
D-11 (A-8)	NIDDM, some under glibendamide, 41 – 68 y, BMI 26.6 ± 2.9	5	16 week	10 g powder in 100 mL water before each meal	30 g/d FS-2	TC	5.95	5.85	5.60
						HDLc	1.05	1.05	1.20
						TAG	3.22	2.60*	1.80*

Cont.

Ref. [†]	Subjects	Sample size	Intervention period	Route of administration	Daily intake	Main results in mmol/L [‡] (baseline, at 4 th week, end point)			
C-7 (D-1)	Hyperlipidemics, 32 – 59 y, BMI 25.3 ± 0.6, usual life style	10	4 week	Kenjin-Salon (tea drink), 1 can (340 g) per meal	15.4 g/d FS-2	TC	5.70	5.40	–
						HDLc	1.10	1.20	–
						TAG	2.32	1.58*	–
C-14 (A-6)	Males with borderline hyper- glycaemia (6.1 – 7.0 mmol/L), 44.4 ± 6.9 y, BMI 25.4 ± 3.3	10	12 week (8-week wash-out)	Soft drink, 1 bottle (100 mL) per meal	29.4 g/d PF-C	TC	5.30	5.30	5.30
						HDLc	1.30	1.35	1.30
						TAG	2.10	1.46*	1.72
D-8 [§]	Mild hypertriglyceridemia (1.7 – 2.8 mmol/L), male, 36.2 ± 10.4 y, BMI 26.6 ± 2.2	9 per group	4 week	Iikoto tea (bottled drink), ½ bottle (250 mL) per meal	16.5 g/d FS-2	TC	5.90	5.65	–
						HDLc	1.30	1.25	–
						TAG	2.22	1.76**	–
D-9	Male with either TC>5.7 mmol/L or TAG>1.7 mmol/L, 46.1 ± 3.0 y, BMI 25.8 ± 0.9	12	12 week	10 g NDP with each meal	30 g/d NDP	TC	6.00	–	5.20**
						HDLc	1.25	–	1.15
						TAG	3.90	–	2.18**

* $p < 0.05$, ** $p < 0.01$ compared with baseline.

FS-2, Fibersol-2 (registered name for a non-digestible corn pyrodextrin); HDLc, high-density lipoprotein-cholesterol; NDP, non-digestible pyrodextrin (Fibersol-2 or Pine Fibre-C); PF, Pine Fibre (registered name for a mixture of 65 % Pine Fibre-C and 35 % maltodextrin); PF-C, Pine Fibre-C (registered name for a non-digestible potato pyrodextrin); TAG, triglycerides; TC, total cholesterol.

[†] The reference identification used in this table corresponds to that assigned originally by Matsutani Chemical Industry Co. Ltd. All these references are published in Japanese and none of these journals was indexed in the Web of Science (Science Citation Index) by August 2002. References: Ref. C-7(D-1), Tokunaga & Matsuoka (1999); Ref. C-13(D-7), Kishimoto *et al.* (2000b); Ref. C-14(A-6), Mizushima *et al.* (2000); Ref. C-16, Shioda *et al.* (2001); Ref. D-4, Nomura *et al.* (1992); Ref. D-6, Kawasaki *et al.* (2000); Ref. D-8, Kajimoto *et al.* (2000); Ref. D-9, (2000a); Ref. D-10, Matsuoka *et al.* (1992); Ref. D-11(A-8), Fujiwara & Matsuoka (1993).

[‡] Original data were expressed in mg/dL and converted to mmol/L using 2.59×10^{-2} and 1.13×10^{-2} as multiplying factors for total cholesterol and triglycerides, respectively (normal values for total cholesterol < 5.20 mmol/L and triglycerides < 2.20 mmol/L).

[§] Placebo-controlled double blind trial.

Fibersol-2 has also been reported to increase stool frequency. A significant increase in stool frequency from 7.3 to 9.3 times during a 10 day period was found in 27 adults who had less than one evacuation per day when they took a commercial drink containing Fibersol-2 (6.25 g/d) in a single blind crossover study (Umekawa *et al.*, 1999). This effect is especially marked in constipated people. Kimura *et al.* (1998) reported a significant increase in stool frequency from 2.5 to 4.5 times per week in female volunteers who had less than 3 evacuations per week, but no change (8.7 to 7.9 times per week) in those volunteers who had more than 7 evacuations per week. In this study, Fibersol-2 (5 g/d) was consumed as part of a commercial jelly. Similar results have been reported using different vehicles for Fibersol-2 (dose: 5 – 8 g/d); cookies (Ogiso *et al.*, 1999), crackers (Shi *et al.*, 2000), flavoured yoghurt drink (Unno *et al.*, 2000), vegetables and fruits mix juice (Tanaka *et al.*, 2000; Unno *et al.*, 2004), peach juice (Yamamoto *et al.*, 2000), vegetable juice (Unno *et al.*, 2001), fruit and vegetable powdered drink (Inaki *et al.*, 1999), AOJIRU powdered drink (Takagaki *et al.*, 2001), cured loin-roll ham (Sato *et al.*, 2000a) and sausage (Sato *et al.*, 2000b).

The Matsutani group led by Wakabayashi studied the acute and chronic effects of a non-digestible potato pyrodextrin (Pine Fibre-C) on the changes in plasma glucose and insulin levels in rats (Wakabayashi *et al.*, 1993; Wakabayashi *et al.*, 1995). To assess the acute effect, they fed six-week-old Sprague-Dawley male rats *ad libitum* with a stock diet for two weeks. Then, after an overnight fast, the animals received an oral load (1.5 g/kg body weight) of different carbohydrates with or without the non-digestible pyrodextrin (0.15 g/kg body weight) (Wakabayashi, 1993). They found a significant reduction in both plasma glucose

(8 – 14 % of the area under the curve, 2 h) and insulin levels (24 – 28 %) in the presence of the non-digestible pyrodextrin, after loading with sucrose, maltose and maltodextrin, but there was no change after the glucose, high fructose syrup or lactose loading (Wakabayashi *et al.*, 1993; Wakabayashi *et al.*, 1995). It is unlikely that these results were due to a delay in gastric emptying or a decrease in the diffusion in the small intestine because this pyrodextrin had low viscosity (Wakabayashi *et al.*, 1995).

Another explanation may lie in the inhibition of intestinal disaccharidases (i.e. sucrase and maltase) and/or the absorption mechanism. Evidence for the inhibition of disaccharides is contradictory. Two reports from the Matsutani group, using rat intestinal mucosa homogenates, showed that Pine Fibre-C did not inhibit the *in vitro* activity of sucrase (Wakabayashi, 1992) or maltase (Wakabayashi *et al.*, 1993), but in another study they showed that Pine Fibre-C did inhibit sucrase activity, using homogenates from rats fed with a Pine Fibre-C enriched diet for nine weeks (Wakabayashi *et al.*, 1992b). Moreover, a Korean group reported an inhibition of sucrase, but not of maltase (Choi *et al.*, 1998).

Regarding the absorption mechanism, it has been also shown, using rat everted intestinal sacs, that the sucrose disappearance from the mucosal medium was not affected by the presence of the non-digestible pyrodextrin. However, the appearance of glucose (from sucrose) in the serosal medium decreased; suggesting that the sucrase activity is not affected, but the transport activity (perhaps SGLT1, but this has not been shown specifically) related to this disaccharidase is (Wakabayashi, 1992). This was further supported by *in situ* sucrose and maltose absorption experiments in rats (Wakabayashi *et al.*, 1993).

To assess the chronic effect of non-digestible pyrodextrins on changes in plasma glucose and insulin levels, three-week-old rats were fed *ad libitum* with a high sucrose diet (65 % sucrose, 25 % casein, 5 % corn oil, mineral and vitamin mixtures) or a pyrodextrin diet (95 % high sucrose diet plus 5 % non-digestible potato pyrodextrin). An oral glucose tolerance test was performed (glucose load: 1.5 g/kg body weight) after eight weeks. Both plasma glucose (11 % of the area under the curve, 2 h) and insulin levels (33 %) had decreased significantly in the rats fed the pyrodextrin diet (Wakabayashi *et al.*, 1995). Thus, there is an improvement in glucose and insulin tolerance in rats after Pine Fibre-C ingestion, however it is not clear what the mechanism is.

Studies in humans have shown a similar picture to those in rats. Wakabayashi *et al.* (1999) reported the effect of several carbohydrates with and without 10 g Fibersol-2 on plasma glucose and insulin levels in 5 healthy men. After 30 min of the ingestion of 100 g sucrose, both plasma glucose and insulin levels increased to a peak (9.4 mmol/L glucose and 37 mU/L insulin). The presence of 10 g Fibersol-2 in the sucrose load significantly reduced both the plasma glucose and insulin peaks by 12 % and 30 %, respectively. A single load of 50 g glucose produced, on the other hand, a peak of plasma glucose and insulin levels at 60 min. However, 10 g Fibersol-2 in the glucose load did not affect the plasma glucose, but decreased the insulin levels by 48 %. Similar improvement in the glucose tolerance was found using meals (Japanese noodle with rice or sweet rolls) instead of the sugars and using drinks with and without Fibersol-2 (Wakabayashi *et al.*, 1999).

Several other single-blinded, placebo controlled and crossover trials either using Fibersol-2 or Pine Fibre-C (5 – 10 g) have shown a similar picture. In all cases, the decrease in the postprandial glucose response (10 – 15 %) was more evident and significant in those subjects whose glucose peaks were above the average (7.5 – 9.3 mmol/L glucose) 30 min after the ingestion of the placebo meal. The non-digestible pyrodextrins were supplied to the subjects as a powder to be dissolved in a glass of water (Maeda *et al.*, 2001) or using several vehicles, such as a tea drink (Tokunaga & Matsuoka, 1999), green tea (Sinohara *et al.*, 1999), soft drink (Mizushima *et al.*, 1999), tofu (Uno *et al.*, 1999), yoghurt (Shioda *et al.*, 2001), soup (Kishimoto *et al.*, 2000b) and boiled rice (Sekizati & Yonezawa, 2001), along with a carbohydrate meal, usually Japanese noodles with rice. However, Wolf *et al.* (2001) using a double blind crossover design found no effect of Fibersol-2 on the glycaemic response of healthy non-diabetics adults after consuming 67.5 g corn syrup solids with or without 16 g of Fibersol-2.

The studies on Fibersol-2 and other pyrodextrins, most of them printed in Japanese, have focused mainly to investigate their laxative effect, improvement in glucose tolerance and decrease in serum triglycerides. Little is known about the effect of Fibersol-2 on SCFA and bacteria in human faeces. Although one study have suggested a possible prebiotic effect with a non-digestible depolymerised corn pyrodextrin (Satouchi *et al.*, 1996).

The main aim of this work therefore was to investigate the effect of a commercial non-digestible pyrodextrin, Fibersol-2, on the faecal SCFA in healthy humans using a double blind, pseudo-randomised pilot trial. The secondary aims were to investigate the effect of Fibersol-2 on the growth of bacteria (Bifidobacteria and

Lactobacilli) and stool frequency and consistency. Additionally, the feasibility of using Fibersol-2 for a longer study as also assessed.

To achieve this goal, a double-blinded crossover trial was used. In this study design, each subject acted both as test and as control, so the variability within subjects is reduced (Jadad, 1998). The main variable measured in this study was SCFA in faeces, specifically the propionate proportion. It can be argued that faecal SCFA does not resemble what really happened during fermentation, because the fermentation in humans occurs in the proximal colon whereas stools have to go through the rest of the colon to be voided. During this transit, SCFA can be absorbed by the mucosa. However, the measurement of stool SCFA was used for this pilot study as a preliminary step to test the feasibility of *in vivo* studies and to see if it was worth doing a larger and more complete study.

The feasibility study was necessary to determine whether subjects will eat the yoghurt preparation in long-term studies, also to investigate whether the dose was suitable or if it causes excessive gastrointestinal symptoms, such as liquid stool consistency and rapid colonic transit. Fibersol-2 is likely to have an osmotic effect. In large doses, it could cause diarrhoea (Satouchi *et al.*, 1993), which would invalidate any health benefits.

6.2. Material and Methods

6.2.1. Sample size

The sample size was calculated using the equation and assumptions described in Chapter 2. The main variable of this human trial was considered the propionate proportion in faeces, because of the results of the *in vitro* study (Chapter 3). Using data from preliminary SCFA analysis, the proportion of propionate was estimated as 16.6 (sd 3.5) %, $n = 4$. An increase of at least 30 % of this value will be considered as a clinically significant difference ($\delta = 5.0$), therefore, a sample size of 16 subjects would be needed for both test and control groups. One of the assumptions used for this calculation was that means would be compared using unpaired t-test, however the crossover design chosen for this study (see below) allowed us to compare means using paired t-test. Hence, a smaller number of subjects should be sufficient.

6.2.2. Subjects

The participants were recruited by word of mouth in the Division of Developmental Medicine, University of Glasgow. The inclusion criteria were healthy male and female subjects between 20 and 65 years old, willing to eat yoghurt three times a day and provide stool samples. Subjects were excluded if they were taking any medication, being on a special diet or having taken any antibiotics in the last three months.

6.2.3. Study design

An outline of the study design is shown in Figure 6.1. The trial was double blind, pseudo-randomised (see below) and crossover in design. The two 8-day intervention periods were carried out with a 3-month wash out period between them. The participants consumed 30 g Fibersol-2 per day mixed with low-fat

natural yoghurt during the test period and the yoghurt alone during the control period. They were asked to consume seven teaspoonfuls of the preparation with breakfast, seven teaspoonfuls with lunch and the rest at the evening meal. They were also asked to keep to their regular diet and life style, except for avoiding a high-fibre intake, which was assessed by keeping a diary (see below).

A fresh faecal sample was supplied by the volunteers at the beginning and at the end of each intervention period. The faecal samples were analysed for SCFA, pH and bacteria quantification. Food intake and stool frequency diaries were also kept by the volunteers. To keep the blindness of the trial, third parties (Sinead Knox and Anna Vogiatzoglou, Master students at the Division of Developmental Medicine) were involved in the subject randomisation and preparation of yoghurts. The ethics committee for non-clinical research involving human subjects of the University of Glasgow reviewed this study.

6.2.4. Randomisation of volunteers

Before the first intervention period of the trial, the participants ($n = 12$) were pseudo-randomised into test and control groups. The pseudo-randomisation was done according to the order in which each volunteer agreed to participate in the intervention: volunteers with odd numbers ate firstly the test yoghurts and then the control ones, whereas participants with even numbers ate the control yoghurt first and then the test one. This code was only known by Miss Knox and Miss Vogiatzoglou and kept in a closed envelope until the last sample was analysed.

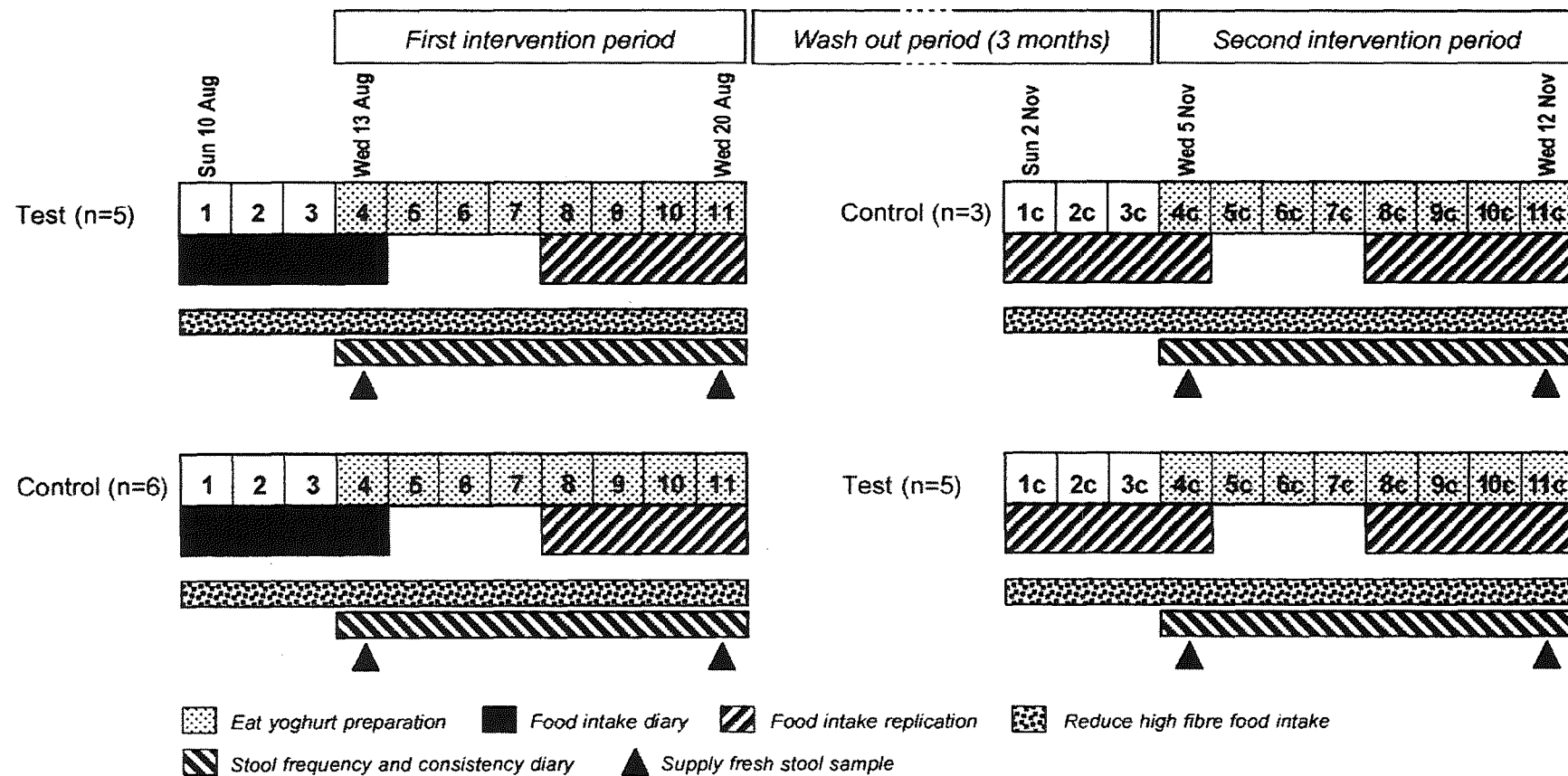


Figure 6.1. Study design.

A summary of the study design to investigate the effect of Fibersol-2 on faecal SCFA in healthy humans is outline. Details are given in the text.

6.2.5. Supplying Fibersol-2 to the volunteers

After the study of some properties of two commercial products, Canary Island gofios and Fibersol-2 in Chapter 5, we selected Fibersol-2 as a suitable pyrodextrin for the human trial. In this section, the choice of a vehicle to supply this carbohydrate to the volunteers is addressed.

6.2.5.1. Choice of vehicle for Fibersol-2

Fibersol-2 was supplied by Matsutani Chemical Industry Co. Ltd. (Itami, Japan) as a white, free flowing powder. The first thought therefore was to provide the carbohydrate in daily dose sachets to be dissolved by the participants in a drink of preference (glass of water, cup of tea or coffee). However, it would be difficult to provide an appropriate placebo and keep the study blinded.

A second option was to use yoghurt as the vehicle. Yoghurt does not contain fibre; therefore, it was unlikely to interfere with any possible action of Fibersol-2. In addition, yoghurt produces a low glycaemic response; reducing any possible interference of the vehicle with the carbohydrate tested as it has been previously shown that Fibersol-2 may reduce postprandial glucose responses in rats (Wakabayashi *et al.*, 1993), although this effect has not yet been established in humans (Wakabayashi *et al.*, 1999; Wolf *et al.*, 2001).

To test whether the volunteers could detect the presence of Fibersol-2 in a mixture with yoghurt (231 g Fibersol-2/kg), the carbohydrate was added to flavoured yoghurt (Vanilla Müller light – virtually fat free yoghurt). Then, nine persons at the Division of Developmental Medicine were asked to taste both the yoghurt and the mixture (using a single blind design) and described if there was any difference in taste between the preparations. Only two people said that the mixture was

sweeter than the yoghurt alone. They were also asked to decide which one they would prefer if they had to eat the preparation everyday for 6 weeks. Three subjects said that they would prefer the mixture; three would prefer the yoghurt and the other three had no preference (i.e. they could eat either preparation). Yoghurt therefore looked a good option to supply the carbohydrate. However, some people expressed their concern about the yoghurt flavour, so we decided to test natural yoghurt as well. For this, Fibersol-2 was mixed with natural yoghurt (Yeo Valley Organic Bio Live natural yoghurt) at different concentrations and twelve subjects were asked to choose (in a single blind design as well) which preparation they like the best. Six subjects preferred the mixture at 300 g/kg, five preferred the mixture at 231 g/kg and one person chose the control (yoghurt alone) as his preferred choice. No-one preferred the mixture at 375 g/kg. Natural yoghurt was therefore chosen as the carbohydrate vehicle in this study.

6.2.5.2. Preparation of cartons

The yoghurt preparations were supplied to the volunteers in half pint, cardboard cartons (Coldstore Packaging Ltd., Kilmarnock, UK). “Safeway low fat natural yoghurt – ideal for cooking” was selected from several natural yoghurts found in the shops around Yorkhill NHS Trust in Glasgow (Table 6.2), because it was the only one with no added probiotic organism (verified with the manufacturer). The presence of probiotic bacteria in the carbohydrate vehicle may alter any possible effect of Fibersol-2 in the colon.

Table 6.2. Natural yoghurt brands found in shops nearby Yorkhill NHS Trust.

Brand*	Added Bio cultures
House of Westphalia Bio garde® – low fat natural yoghurt with added calcium	Yes
Onken Natural Biopot set yoghurt	Yes
Pakeeza live natural yoghurt	Yes
Rowan Glen naturally Scottish bio natural – Scottish natural yoghurt	Yes
Safeway low fat Bio natural yoghurt – ideal for cooking	Yes
Safeway low fat Natural yoghurt – ideal for cooking	No
Yeo Valley Organic Bio Live natural yoghurt	Yes

* Information as appears on the label.

The carton preparations were made isoenergetic, given a total energy intake of 276 kJ per carton. Test cartons were prepared by blending (1 min at full speed using a Kenwood blender, model A516-517) 30 g Fibersol-2, which has an energy value of 2.2 kJ/g (Tsuji & Gordon, 1998), with 70 g yoghurt, which has an energy values of 3.0 kJ/g (product label). The sheering force produced during the blending made the yoghurt become less viscous. The control carton, therefore, was prepared blending 92 g yoghurt under the same conditions. The nutritional information of both preparations is given in Table 6.3. The cartons were prepared and supplied to the participants by Miss Sinead Knox (in the first intervention period) and Miss Anna Vogiatzoglou (in the second intervention period).

Table 6.3. Nutritional information of the carton preparations*.

	Test carton	Control carton
Energy (kJ)	276	276
Protein (g)	4.6	6.1
Carbohydrate (g)	6.2	8.1
Fat (g)	0.8	1.0
Fibre (as Fibersol-2; g)	26.9 [†]	Nil

* Based on the nutritional information on the yoghurt label.

[†] Calculated using a value of 89.8 % of non-digestible fraction for Fibersol-2, after a sequential hydrolysis *in vitro* with human salivary α -amylase, artificial gastric juice, porcine pancreatic α -amylase and rat small intestine mucosa (Tsuji & Gordon, 1998).

6.2.6. Bacteria quantification – semi-quantitative method

A semi-quantitative bacteriological method (Giaffer *et al.*, 1991) was used to estimate the number of Bifidobacteria and Lactobacilli present in the stool. This method is quicker and less skillful than the quantitative method described in Chapter 5. It is therefore the choice when a large number of faeces are analysed together. Briefly (Figure 6.2), a small amount of stool or a tenfold diluted slurry was plated directly onto Beerens and Rogosa plates (inoculum well) using a sterile 2 mm wire loop and standard plating techniques (Gillies & Dodds, 1976). From the inoculum well, four sets of streaks were made, flaming the loop between each set. The plates were placed into an anaerobic jar and incubated for 72 h at 37 °C.

A semi-quantitative score between 1 and 5* was given for each sample depending on how well the organism grew on the selective agar plates. If only a few colonies grew on the inoculum well, on the first set of streaks, on the second set of streaks and so on until the forth set of streaks; a score of 1, 2, 3 and so on until score 5 was assigned, respectively. Additionally, if a large number of colonies grew on the inoculum well and on the respective set of streaks (i.e. colonies along the whole streak length rather than one or two colonies), the score was upgraded. This was denoted adding a star (*) to the score numbers. Stool samples for the end of intervention day (after) were diluted tenfold (1 g stool was mixed well in 10 mL pre-boiled, pre-reduced with OFN water) because of the baseline day (before) scores were high. The scores, therefore, may range between 2 and 6* for end of intervention day data. According to Giaffer *et al.* (1991), a change in the score by one unit corresponded, in most cases, with a change in the colony count by $\text{Log}_{10} 1$, beginning with $\text{Log}_{10} 5.4$ for score 1.

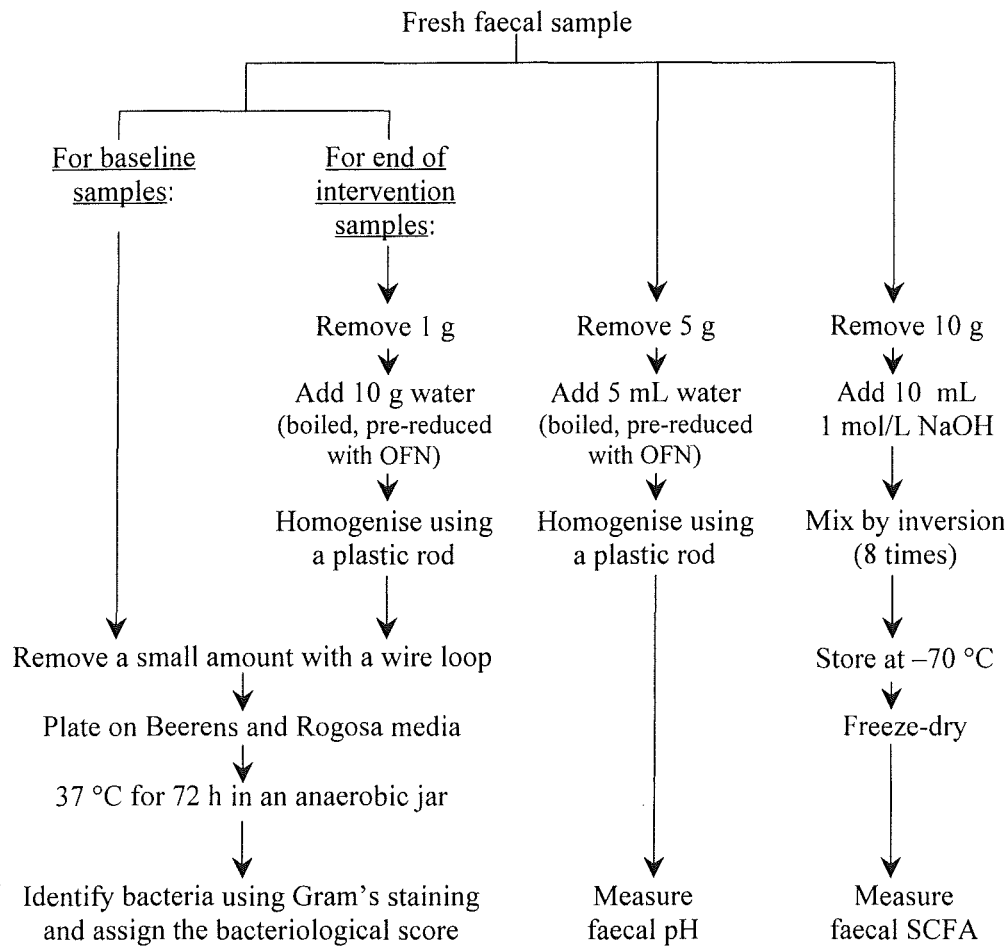


Figure 6.2. Processing of faecal samples.

A summary of the protocols to measure the bacteriological score, pH and SCFA in human faeces is outlined. Details are given in the text. OFN, oxygen free nitrogen.

Representative colonies were picked off from the corresponding set of streaks to confirm their identity. The identity of the colonies was assessed by Gram's staining only, looking at Gram-positive, branched rod-shape cells on Beerens plates for Bifidobacteria and Gram-positive rod-shape cells on Rogosa plates for Lactobacilli.

For Gram's staining, cells were harvested from Beerens or Rogosa plates, transferred to a clean microscope slide and suspended in a water drop. Cell preparations were air-dried and then heat-fixed by briefly passing the slide through the flame.

Gram's staining was carried out as follows (Gillies & Dodds, 1976): heat-fixed cells were immersed in methyl-violet stain (30 parts of 1 % methyl-violet 6B solution and 8 parts of 5 % sodium bicarbonate solution) for 5 min and washed with tap water. Cells were then immersed in iodine solution (2 g iodine in 10 mL 1 mol/L sodium hydroxide and 90 mL water) and washed with tap water after 2 min. De-stained was done quickly immersing the cells in acetone and washing again with tap water. Counterstaining was made by immersing heat-treated cells in basic fuchsin stain (0.05 % basic fuchsin solution) for 30 s and washing again with tap water. Cell films were then blotted and allowed to dry in air before viewing at x1000 magnification on a Carl Zeiss microscope (Germany).

6.2.7. Faecal pH analysis

Faecal pH was measured in slurry prepared by homogenising by hand 5 g of faeces with 5 mL water (pre-boiled, cooled to room temperature and kept in an OFN atmosphere until used) (Figure 6.2).

6.2.8. Faecal SCFA analysis

Faecal SCFA were measured in dried stool samples using the method described in Chapter 2. On the day of the faeces collection, 10 g stools were placed in a 30 mL specimen vial, 10 g of 1 mol/L NaOH solution were added to bring pH to more than 9, mixed by inversion (8 times) and stored at -70°C . After all faeces samples were collected, they were freeze-dried and homogenised to powder (Figure 6.2). To analyse the samples, between 50 and 100 mg dried powder (actual weight recorded) were dissolved in 0.8 mL water, then 0.1 mL internal standard and 0.1 mL concentrated orthophosphoric acid were added. The ether extraction and the rest of the analysis were carried out as described in Chapter 2. Total SCFA was expressed as mmol/kg of dried weight faeces.

6.2.9. Food intake

A food intake diary (see Appendix 4) was given to the participants to record their food intake for three consecutive days (Sunday, Monday and Tuesday) before and on the day of supplying the first faecal sample (Wednesday). The same diary was used by the volunteers to replicate their meals during the three consecutive days before and on the day of supplying the rest of the faecal samples (Figure 6.1). The information recorded in the diaries was also used to estimate the dietary fibre intake using the software Diet 5 (Univation Ltd., Aberdeen, UK) to check the compliance with the request of avoiding high fibre foods during the study.

6.2.10. Stool frequency and consistency

Stool frequency and consistency was recorded by the participants using a diary (see Appendix 4) during each intervention period (Figure 6.1). The stool frequency was calculated as the number of evacuations, during the first 7 days of the intervention, divided by 7 and expressed as day^{-1} . The last day of the

intervention was not included because the subjects were asked to stop recording data after they supplied the faecal sample (usually in the morning).

The stool consistency, on the other hand, was assessed against a simple scale. The participants were asked to describe the consistency of the faeces at every evacuation by using one of the following five categories: hard pellet, solid stool, soft formed, porridge like or liquid. They were also asked to comment on whether the faeces were easy or hard to pass. These data, which were also collected during the first 7 days of the intervention, were pooled together in control and test groups and the frequency of each category within each group was calculated.

6.2.11. Statistical analysis

In a crossover design, it is important to consider whether there is an effect due to the order in which the subjects are test or control (Altman, 1991), i.e. if subjects who ate Fibersol-2 first and then control behave differently than the subjects who ate control first and then Fibersol-2. To evaluate the order effect, a general linear model was applied to faecal SCFA and pH (continuous variables) and an ordinal logistic regression was applied to the bacteriological scores (categorical variables). In all the regression analyses, subject (11 levels: one per each volunteer), treatment (2 levels: control and test) and order (2 levels: first and second intervention period) were used as both model factors and covariates. These analyses showed that there was no order effect for faecal SCFA ($p > 0.15$), faecal pH ($p > 0.05$) or bacteriological scores ($p > 0.65$). As a result, the subject data were re-arranged as control and test groups and the effect of the intervention was carried out by evaluating the difference “after” – “before”, in both test and control groups, using paired t-test (for continuous variables) and Wilcoxon signed rank test (for categorical variables).

For the stool frequency data, the test group was compared with the control group using a paired t-test. On the other hand, the stool consistency data were evaluated using the chi-square for trend test, calculated by hand using the procedure described by Altman (1991). This test is more appropriate than the standard chi-square test when comparing frequencies among groups that have an ordering (e.g. from hard pellet to liquid stools). To carry out the analysis, the five categories described above (hard pellet, solid stool, soft formed, porridge like and liquid) were pooled into three categories (hard pellet-solid stool, soft formed and porridge like-liquid) to give adequate expected numbers in all cells of the analysis (Altman, 1991).

6.3. Results

6.3.1. Subjects

Thirty-one subjects who met the inclusion criteria were asked to participate, but only 12 subjects (5 females and 7 males) were willing to do so. From them, 11 subjects began the first intervention period, because one male subject dropped out due to a cold during the first period and he was out of town by the time of the second period. During the second intervention period, one male (test group) and two female (control group) subjects also dropped out. None of the dropouts for the second intervention period was due to secondary effects from the first period. The reasons for these dropouts were: participating in another human trial (male subject) and out of town by the time of the intervention (both female subjects). Therefore, 5 females and 5 males, age 33.5 (range 24, 46) years old, were in the test group ($n = 10$) and 3 females and 6 males, age 33 (range 28, 46) years old, were in the control group ($n = 9$). The two groups were similar in age, but there

were twice as many male subjects in the test group than there were in the control group.

Subjects were asked to keep their dietary fibre intake as low as possible during the days before the beginning and the end of the two interventions. The dietary fibre intake was the same for both groups: 11.4 (range 8.6, 16.6) g/d for the test group and 11.4 (range 10.0, 16.6) g/d for the control group. However, there were two subjects, whose fibre intake was high (15.7 and 16.6 g/d). The average fibre intake in Scotland is 11-12 g/d (Scottish Office, 1993).

6.3.2. Faecal SCFA and pH

Faecal total SCFA, individual molar proportion and pH values before and after intervention with Fibersol-2 are shown in Table 6.4.

Total SCFA decreased on average -99 (95 % confidence interval (C.I.) from -417 to 220) mmol/kg and -234 (95 % C.I. from -397 to -71; $p = 0.011$) mmol/kg for test and control, respectively (Table 6.5). Individual variations are shown in Figure 6.3. In the test group, 3/10 subjects had higher total SCFA in contrast to 1/9 subjects in the control group, after the intervention.

The SCFA profile changed very little on average. Acetate proportion did not change on average in the test group (95 % C.I. from -4.3 to 4.2 %). However, there was a small but significant ($p = 0.025$) increase in the control group (5.7 (95 % C.I. from 0.9 to 10.6) %) (Table 6.5). Individual data for acetate are shown in Figure 6.4. In the control group, 7/9 volunteers had higher proportion of acetate after the intervention. Propionate on average slightly increased (0.6 (95 % C.I. from -2.0 to 3.2) %) in test group, but slightly decreased (-1.0 (95 % C.I. from -3.2 to 1.1) %) for control (Table 6.5). Figure 6.5 shows the individual variation. In

the test group, 5/10 subjects had higher propionate proportion, similar to the control group, where 4/9 volunteers had a higher proportion of propionate after the intervention.

Table 6.4. Faecal total SCFA, individual molar proportion and pH before and after intervention with 30 g/d Fibersol-2 for one week.

	Test		Control	
	Before (n = 10)	After (n = 10)	Before (n = 9)	After (n = 9)
Total SCFA (mmol/kg) [†]	1037 ± 384	938 ± 316	1026 ± 286	792 ± 308*
SCFA molar proportion (%)				
Acetate	66.4 ± 5.6	66.3 ± 3.5	60.6 ± 8.0	66.3 ± 5.3*
Propionate	14.0 ± 3.6	14.6 ± 2.8	16.0 ± 5.6	15.0 ± 4.6
Butyrate	13.1 ± 3.3	13.5 ± 3.4	16.3 ± 2.8	11.6 ± 2.7*
iC4–C5	6.6 ± 3.0	5.6 ± 2.2	7.1 ± 3.8	7.2 ± 3.4
Faecal pH	6.7 ± 0.6	6.6 ± 0.5	6.6 ± 0.6	6.8 ± 0.5

Values are mean ± standard deviation

* $p < 0.05$ compared with "Before" in both test and control groups (paired t-test).

[†] Dry matter basis.

Table 6.5. Mean difference and 95 % confidence intervals for the mean difference for the variables shown in Table 6.4.

	Test		Control	
	Mean difference (n = 10)	95 % C.I.	Mean difference (n = 9)	95 % C.I.
Total SCFA (mmol/kg)	-99	-417, 220	-234	-397, -71
SCFA molar proportion* (%)				
Acetate	0	-4.3, 4.2	5.7	0.9, 10.6
Propionate	0.6	-2.0, 3.2	-1.0	-3.2, 1.1
Butyrate	0.4	-2.3, 3.1	-4.7	-7.6, -1.9
iC4–C5	-1.0	-2.2, 0.3	0	-1.7, 1.7
Faecal pH	-0.1	-0.4, 0.2	0.3	-0.1, 0.6

* On average, there was a decrease in acetate (6.7 units; $p = 0.031$) and an increase in both propionate (1.8 units; $p = 0.26$) and butyrate (5.8 units; $p = 0.0004$) molar proportion difference due to the intervention (general linear model).

On average, butyrate slightly increased (0.4 (95 % C.I. from -2.3 to 3.1) %) for test, but decreased significantly (-4.7 (95 % C.I. from -7.6 to -1.9) %; $p = 0.0051$) for control (Table 6.5). Individual values are shown in Figure 6.6. Branched SCFA on average slightly decreased (-1.0 (95 % C.I. from -2.2 to 0.3) %) for test and did no change (95 % C.I. from -1.7 to 1.7 %) for control (Table 6.5). Figure 6.7 shows the individual variations.

No significant difference was found in culture pH in either group (Table 6.5). Individual variations are shown in Figure 6.8.

6.3.3. Effect on bacteria growth

Table 6.6 shows the effect of Fibersol-2 on the bacteriological scores. Individual variation for Bifidobacteria and Lactobacilli scores are shown in Figure 6.9 and Figure 6.10, respectively. Bifidobacteria scores increased significantly ($p = 0.006$) for the test and did not change for the control groups. In fact, all subjects had higher scores in the test group after the intervention in contrast to 5/9 subjects in the control group. Additionally, Lactobacilli scores also increased significantly ($p = 0.006$) for the test group and decreased slightly for the control group. In the test group, all participants had higher scores after the intervention in contrast to 4/9 volunteers in the control group.

Table 6.6. Bacteriological scores before and after intervention with 30 g/d Fibersol-2 for one week.

	Test		Control	
	Before (n = 10)	After (n = 10)	Before (n = 9)	After (n = 9)
Bifidobacteria	4 (3, 4*)	5 (4, 5) [†]	4 (3*, 4*)	4* (4, 5)
Lactobacilli	2* (1*, 3)	3 (2*, 4) [†]	3 (1*, 3*)	2* (2*, 4)

Bacteriological score values are median (range). See section 6.2.6 for the score description.

[†] $p < 0.01$ compared with "Before" in both test and control groups (Wilcoxon signed rank test).

6.3.4. Stool frequency and consistency

Stool frequency and consistency were compared only in those subjects who took part in both arms of the trial. On average, stool frequency increased, although not significantly, from 1.11 (SD 0.31) day⁻¹ for the control group to 1.27 (SD 0.61) day⁻¹ for the test group. In fact, it only increased in 2/8 subjects (Figure 6.11). On the other hand, Fibersol-2 produced a significant change ($p = 0.0001$; chi-square test for trend) in the stool consistency towards a softer stool (Figure 6.12). In fact, only 18/49 of the hard pellet-solid faeces, but 14/16 of the porridge like-liquid faeces, was reported in the test group. In addition, the test group described 73/112 and 1/6 of the evacuations as easy-to-pass and hard-to-pass, respectively. One subject reported diarrhoea that settled after the fourth day of the intervention with Fibersol-2. This subject actually had 7/15 liquid evacuations during this period.

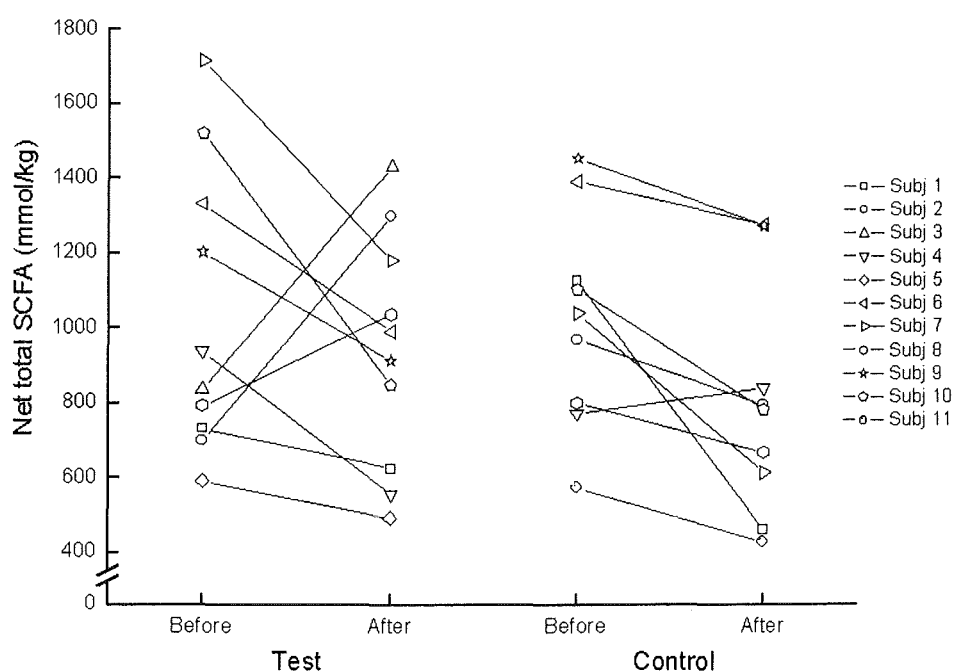


Figure 6.3. Faecal total SCFA for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

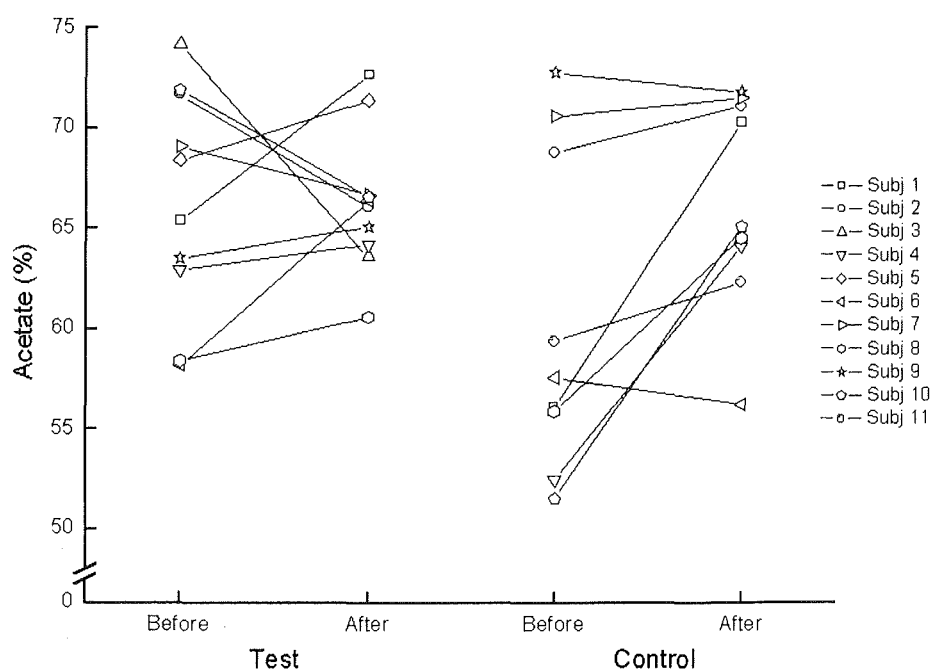


Figure 6.4. Faecal acetate proportion for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

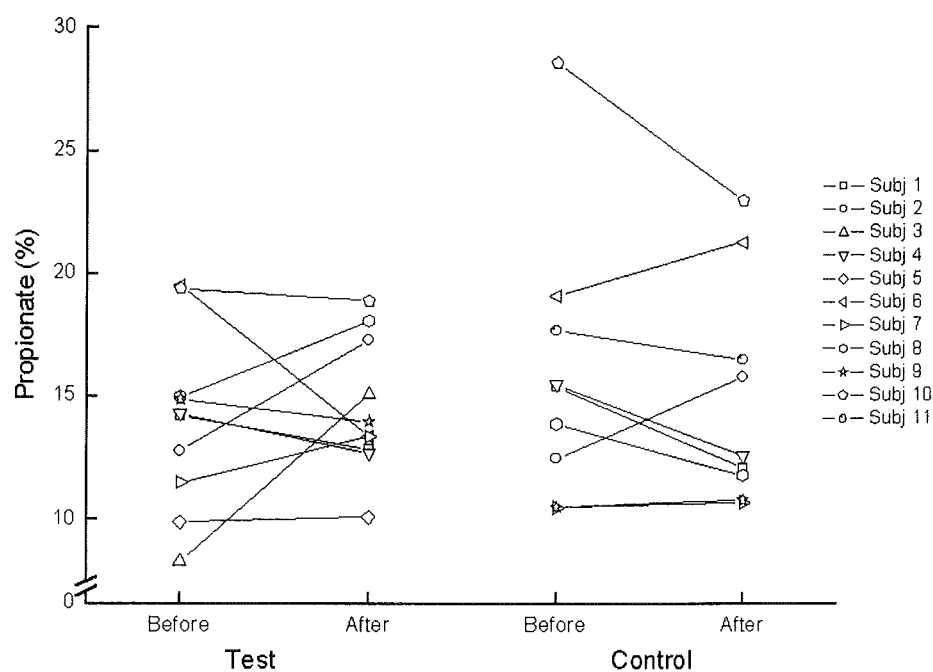


Figure 6.5. Faecal propionate proportion for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

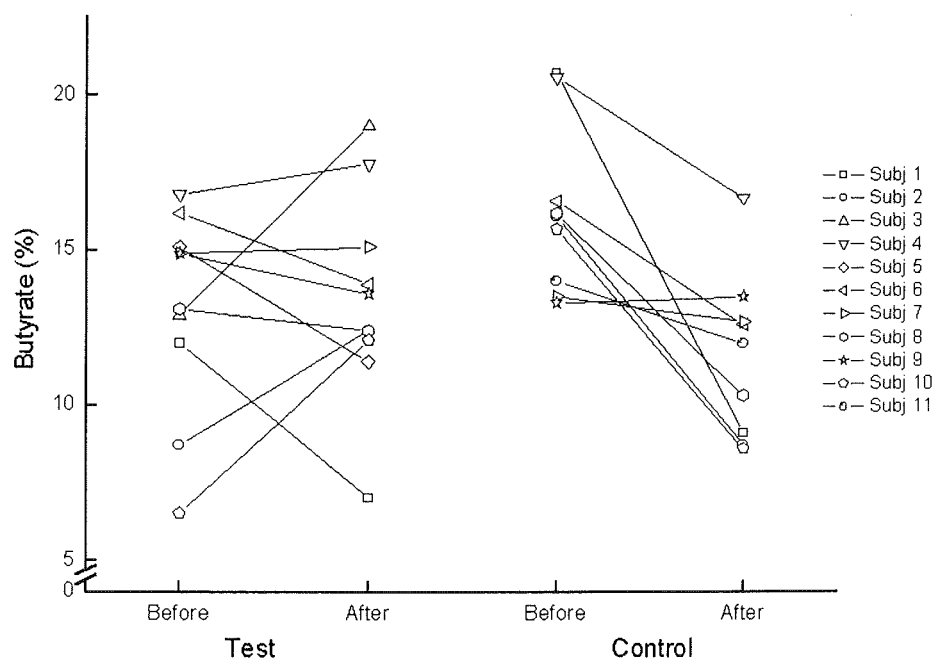


Figure 6.6. Faecal butyrate proportion for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

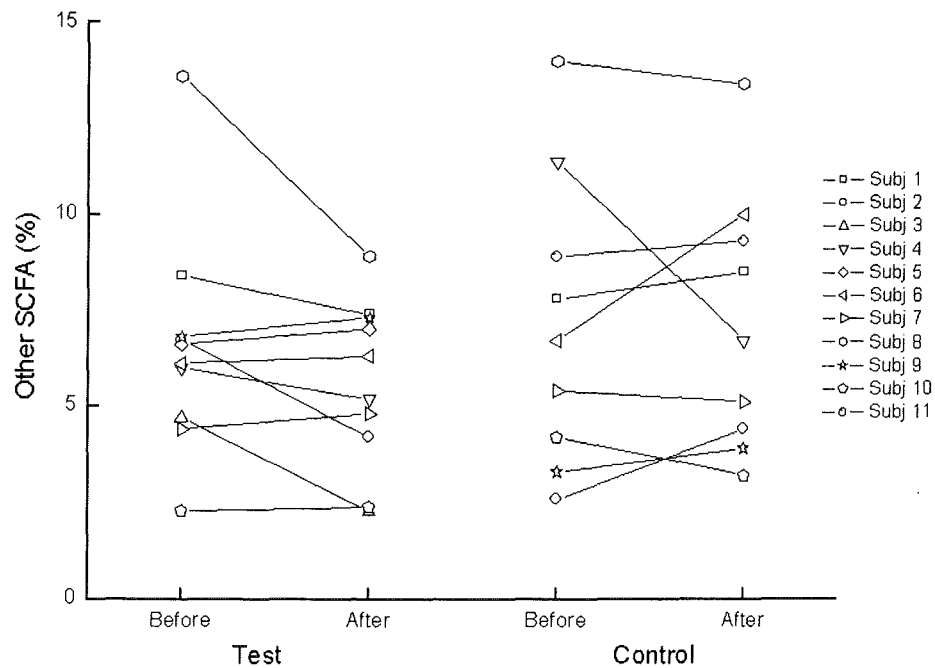


Figure 6.7. Other faecal SCFA (iso-butyrate, iso-valerate and valerate) proportion for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

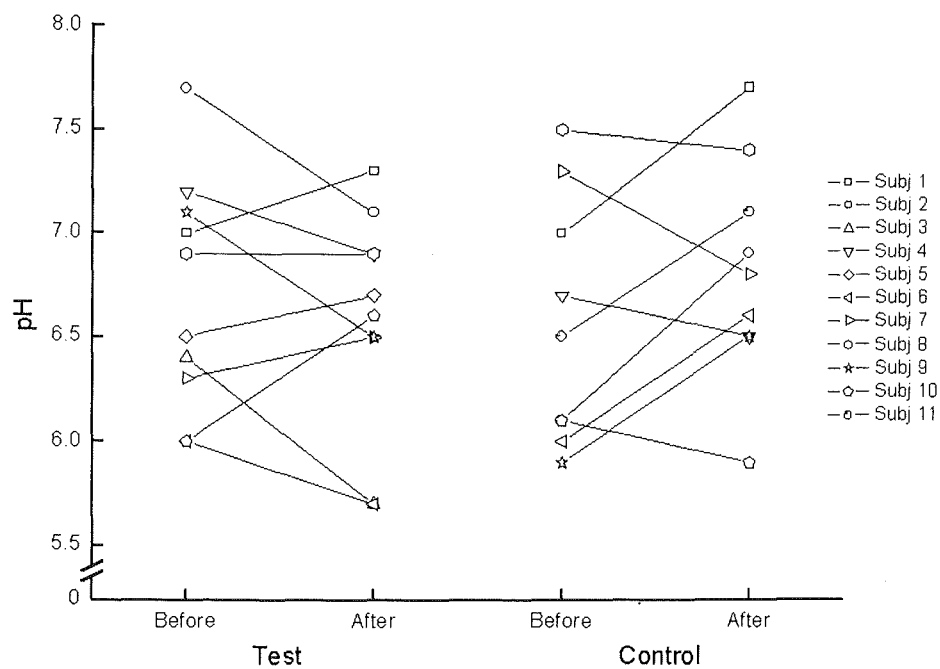


Figure 6.8. Faecal pH for each subject before and after intervention with 30 g/d Fibersol-2 for one week.

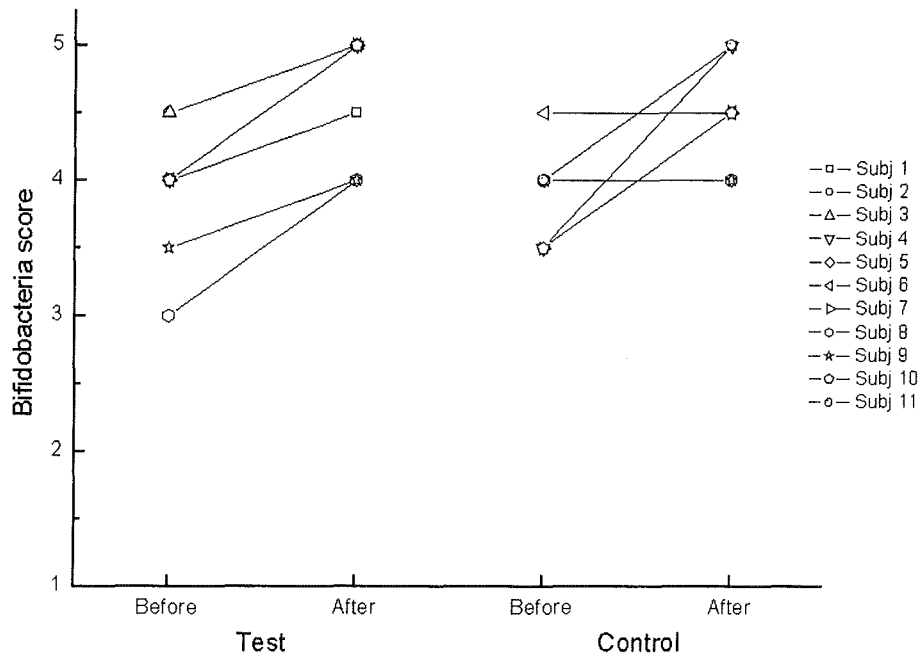


Figure 6.9. Bifidobacteria scores for each individual before and after intervention with 30 g/d Fibersol-2 for one week.

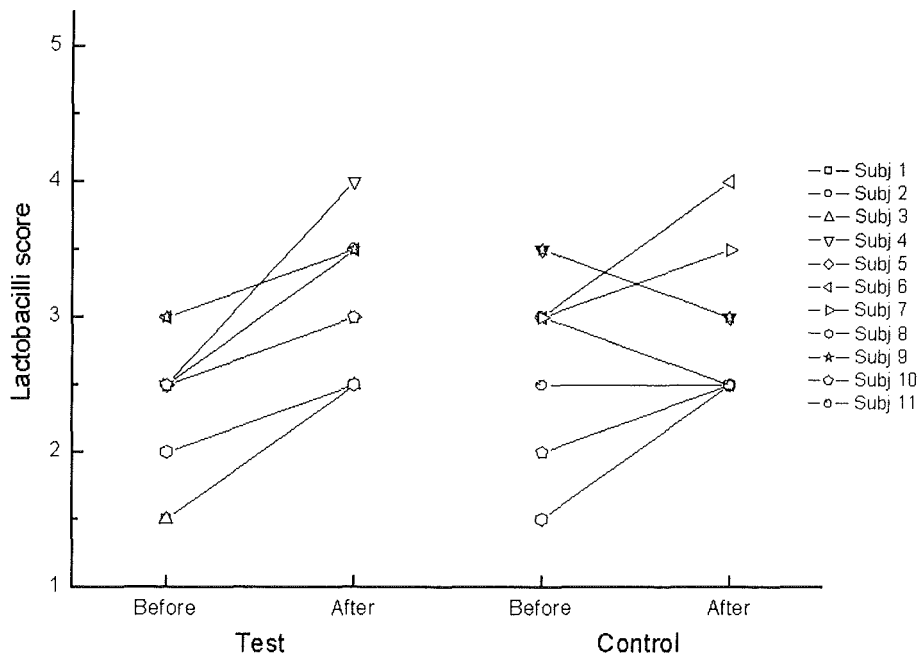


Figure 6.10. Lactobacilli scores for each individual before and after intervention with 30 g/d Fibersol-2 for one week.

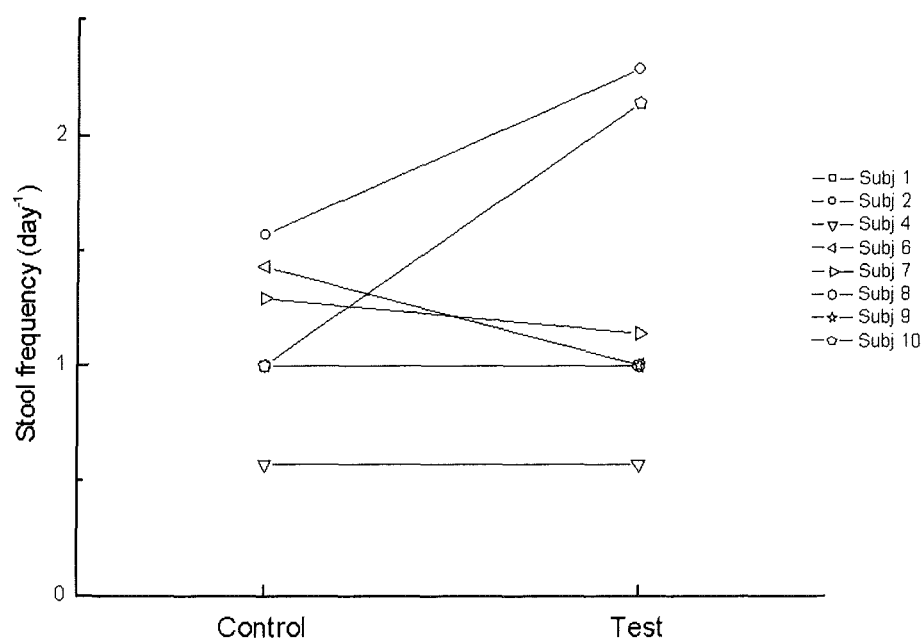


Figure 6.11. Stool frequency for each individual during intervention with and without 30 g/d Fibersol-2 for one week.

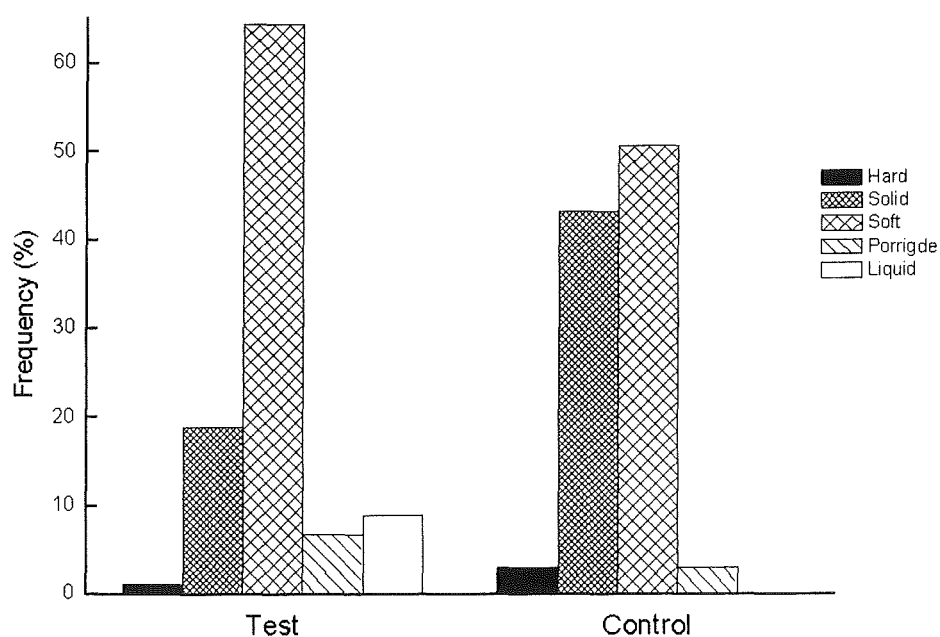


Figure 6.12. Stool consistency for test and control groups during intervention with or without 30 g/d Fibersol-2 for one week.

A total number of 90 and 67 evacuations were reported for test and control groups, respectively.

6.4. Discussion

As it was pointed out at the beginning of this chapter, Trial 1 (plasma triglyceride study) had severe problems with recruitment of volunteers. Only two people have completed the study, but no data have been analysed yet. Thus, we do not know if Fibersol-2 decreases serum triglyceride levels in our study population. A review of the Japanese literature on Fibersol-2 has showed that this may be the case (Table 6.1). If Fibersol-2 is shown to lower serum triglyceride, it may prove to be a viable product that could be added to common foods with little or no disruption to taste and texture. It could be possible to produce a choice of foods with added Fibersol-2 and provide them to the public. Indeed, there are several commercialised products in the Japanese market that contain Fibersol-2, such as tea, juices, jelly, cookies, yoghurt, tofu, soups, among others (a full list is given in Chapter 5). A choice of food with Fibersol-2 added, may help individuals at risk of coronary heart disease to lower their serum triglyceride levels without having to drastically change their diet, possibly helping to lower the incidence of cardio-vascular disease in the population. Indeed, similar trials using other fibre sources have had largely positive effects (Romero *et al.*, 1998). Trial 1 is still running and a summary of the study design is presented in Appendix 3.

The effect of 30 g/d of Fibersol-2 on faecal SCFA and pH after the one-week intervention period (Trial 2) was negligible in the test group. None of the changes observed during the *in vitro* fermentation of the starch pyrodextrins (Chapter 3) were observed *in vivo*. Fibersol-2 differs from the starch pyrodextrins studied in Chapter 3. The commercial product has a lower molecular weight than the pyrodextrins. In fact, during the pre-digestion process carried out before the *in vitro* incubations of the pyrodextrins, a dialysis step washed out the small

compounds (MW < 2000). Fibersol-2 in contrast has a molecular weight of 2000. Although both potato pyrodextrin and Fibersol-2 showed evidence that suggest the presence of the β glucosidic bonds, it seems that the most abundant of these non-starch bonds was $\beta(1\rightarrow2)$ in potato pyrodextrin and $\beta(1\rightarrow3)$ in Fibersol-2. Despite the difference in the chemical structure of both preparations, the *in vitro* fermentation profile of Fibersol-2 was similar to the profile of the pyrodextrins. On the other hand, faeces are not the best models to study the changes in SCFA profile. Most of the SCFA are produced in the human proximal colon and may be absorbed during their transit to the rectum. Hence, measurements of SCFA in faeces may not reflect their production in the proximal colon. Better methods are needed to determine SCFA production *in vivo* and particularly the amounts of propionate reaching the liver. Not enough propionate gets into the systemic blood to allow colonic flux studies at present. However, such studies have been performed with acetate production (Simoneau *et al.*, 1994; Lifschitz *et al.*, 1995; Kien *et al.*, 1996) and as detection levels for propionate improve, this technique may be used in the future.

Interestingly, there was an effect of the yoghurt preparation in the control group. After the one-week intervention, volunteers acting as control had significantly lower total SCFA, an increase in acetate and a decrease in butyrate proportions. The cause of this behaviour is not known.

Lactobacilli were defined as Gram-positive, rod-shape cells growing on the selective media, Rogosa. Bifidobacteria were defined as Gram-positive, branched rod-shaped cells growing on the Beerens media. A further identification of the subspecies would require the use of biochemical tests (Gillies & Dodds, 1976).

Fibersol-2 significantly increased both Bifidobacteria and Lactobacilli scores (Table 6.6). Pyrodextrins similar to Fibersol-2, but with lower molecular weight, were also fermented by several species of Bifidobacteria, Lactobacilli and Bacteroides (Satouchi *et al.*, 1996). These results suggest that Fibersol-2 may have an effect promoting the growth of both Bifidobacteria and Lactobacilli. Using a non-digestible depolymerised corn pyrodextrin, Satouchi *et al.* (1996) reported an increase in Bifidobacteria yet a decrease in Lactobacilli populations when seven male volunteers were fed with 10 g/d of the depolymerised pyrodextrin for two weeks. However, it was not possible to determine in our study if Fibersol-2 selectively increased the proportions of these two groups of bacteria, which would define Fibersol-2 as prebiotic, because we did not look at any other bacteria. Fibersol-2, therefore, may have just increased the growth of all the anaerobic bacteria.

Stool frequency did not change due to Fibersol-2. Several studies have shown an increase in stool frequency only with constipated people (i.e. less than 3 evacuation per week) (Kimura *et al.*, 1998; Umekawa *et al.*, 1999). This was not the case in our study where the stool frequency was slightly more than one evacuation per day on average. However, two participants did report an increase in stool frequency: for Subject 10 the frequency doubled and for Subject 2 increased 50 % (Figure 6.11). This commercial pyrodextrin changed the stool consistency towards a softer stool. A similar tendency was reported using equivalent amounts of Pine Fibre given in a single dose (Satouchi *et al.*, 1993).

The study design was accepted by the volunteers. Even though there were dropouts for the second intervention period, none of them can be attributable to

the yoghurt preparation. The low-fat natural yoghurt was successful as the vehicle for Fibersol-2. At the end of the second intervention period, the subjects who took both yoghurt preparations were asked if they preferred either of the two preparations. Six out of eight subjects preferred the yoghurt with Fibersol-2, one preferred the yoghurt without the carbohydrate and the other subject had no preference at all. The preference for the mixture of Fibersol-2 with yoghurt may be due to the slight sweetness Fibersol-2 gave to the low-fat natural yoghurt used.

The dose of Fibersol-2 used (30 g/d) had no major side effects. Only one subject complained of diarrhoea for the first three days, but the dose was well tolerated over the length of the intervention. These results suggest that the study design used for this pilot experiment would be suitable for a long-term clinical study, such as the study designed to investigate the effect of pyrodextrins on plasma triglycerides levels in men (Trial 1).

In conclusion, Fibersol-2 had little effect on faecal SCFA. However, it showed some potential as a prebiotic agent. Further studies are needed to establish the effect of this non-digestible pyrodextrin on plasma lipids and other risk factors for cardio-vascular disease. The *in vivo* fermentation of pyrodextrins cannot be adequately studied in humans using present techniques.

Chapter 7.

General discussion and Conclusions

During the time for this PhD, I have attempted to study starch pyrodextrins from three different angles: (1) how they are fermented in an *in vitro* model of the human large intestine, (2) how does their chemical structure influence their fermentation and (3) how does this fermentation affect the physiology of human beings.

I have been interested in starch pyrodextrins for a few years before the beginning of this PhD. As a junior lecturer at the Universidad Central de Venezuela in Caracas, I had the opportunity to work under the supervision of Dr Juscelino Tovar at the time one of his students discovered that pyrodextrinising unripe plantain starch led to a remarkable decrease in the enzymatic availability of the starch. We therefore designed a research plan that led us to the characterisation of both the physicochemical properties and *in vitro* digestibility of pyrodextrins produced from several plant sources and with different pyroconversion conditions. A decrease in the enzymatic availability of the pyroconverted starches was recurrent in all our studies. As a matter of luck (or destiny!), Dr Christine Edwards went to Venezuela to share her expertise on the study of colonic fermentation using simple *in vitro* models of the human colon. It was indeed a golden opportunity for me to go further and study what happens with those pyrodextrin fractions that may escape the small intestine and reach the colon.

Hence, my first approach in this PhD was to use an *in vitro* batch model with human faecal bacteria (Edwards *et al.*, 1996) to study the fermentation of pyrodextrins and their native starches. It was established that the changes that the starch polymers undergo during the pyrodextrinisation process did change the proportions of SCFA produced. The fermentation of pyrodextrins resulted in a higher proportion of propionate compared with the native starch from the same botanical source. Moreover, butyrate production, an important characteristic of starch fermentation (Edwards & Rowland, 1992), was maintained.

There are potential health benefits associated with increased proportion of propionate along with a parallel decrease in the proportion of acetate. Some human studies have suggested that a higher propionate to acetate ratio may reduce both plasma triglycerides (Wolever *et al.*, 1995) and hepatic cholesterol synthesis (Wolever *et al.*, 1991). Whether colonic fermentation of pyrodextrins produces enough propionate to reduce plasma triglycerides and/or cholesterol *in vivo* and whether this reduction helps to decrease the incidence of cardiovascular disease has yet to be established. However, it is an interesting hypothesis that deserves further investigation.

The exact reason for this change in the SCFA profile is unclear. The increased propionate production may be due to the increased solubility or the presence of the non-starch bonds in the pyrodextrins over the native starches. The activity of certain bacteria, which can cleave β bonds, may be increased. Similar proportions of propionate were produced *in vitro* by fermentation of soluble glucose oligosaccharides, which had various glycosidic linkages (Flickinger *et al.*, 2000), but β bond containing insoluble glucose polymers such as cellulose (Velázquez *et*

al., 2000) and β -glucan (Karppinen *et al.*, 2000) also produced a higher proportion of propionate compared to α -glucans.

Of course, the studies described in Chapter 3 were carried out in *in vitro* batch cultures, which have severe limitations and may not reflect what occurs in the human colon *in vivo*. However, it is very difficult to measure colonic fermentation in the intact human colon especially in the proximal colon where most fermentation normally occurs (Edwards & Rowland, 1992). The batch culture has no pH control and a low pH may affect SCFA proportions (Edwards *et al.*, 1985) with an increased production of propionate. However, the final pH in the *in vitro* cultures was in fact in the opposite direction to the increased propionate production. In other words, the final pH of the pyrodextrin cultures was higher than that of the native starches. Therefore, pH does not seem to be a factor in the increased proportion of propionate.

Having searched the published literature on the structure of carbohydrates and after discussions with chemists, I decided to use NMR spectroscopy to further elucidate the structure of starch pyrodextrins from various plant sources. The literature had suggested that very little was confirmed about the branching structure of the pyrodextrin molecule produced in an acidic environment. Although there was some evidence of glucose-glucose bonds not present in starch (Geerdes *et al.*, 1957; Ohkuma *et al.*, 1993a; Ohkuma *et al.*, 1993b), it was not established whether these were α - or β -bonds, but there were strong suspicions that anhydro-glucose (Theander & Westerlund, 1987), and more recently, a glucosyl-cation (Lowary & Richards, 1991), were involved in the transglucosidation reactions, which would produce these non-starch bonds.

Methylation analysis had determined that 1→3 and 1→2 bonds were present. Since anhydro-glucose has a β conformation, it could be predicted that these new bonds were β bonds. However, this had not been firmly established. The NMR analysis I carried out aimed to confirm the presence of β bonds and to gain more evidence of the carbon linkages. However, despite initial plans and hopes for the NMR study, it became clear that the evidence that could be gained was limited by the complex nature of the branching structure and the limited published data on the signals from comparable bonds in other molecules. This limited the interpretation of the NMR spectra but did allow some initial data in support of the presence of β bonds and possibly the $\beta(1\rightarrow2)$ linkage. The published literature suggested that the anhydro-glucose was attached to one end of the polymer molecule (Theander & Westerlund, 1987), the NMR spectra showed the presence of the anhydro-glucose monomers i.e. not attached to the polymer. After the dialysis of the small molecules (MW < 2000), these signals were lost from the pyrodextrin preparation, again confirming that much of the anhydro-glucose was not attached to the polymer.

To fully establish the structure of the pyrodextrins by NMR was not feasible in the time of this PhD. It would require much work to isolate individual small molecules rich in particular bonds which would give a higher signal to noise ratio for individual linkages. This would need extensive fractionation of the pyrodextrin preparation, separation and purification based on molecular weight and other properties and, finally, if enough material remained after this long and material consuming process, detailed two-dimensional NMR spectroscopy. There would be no guarantee that this would result in sufficiently clear NMR spectra to fully establish the bonds and structure. Indeed, this approach would only be possible for

the low molecular weight molecules produced during pyrodextrinisation and would not be possible with the polymeric molecules. Another main limitation, apart from the time taken to purify the molecules, is the time taken for the NMR scans of a polymer molecule. The smaller molecules require a "shorter" scan time. The purification of the small molecules could be carried out using activated charcoal columns, but this would require another three years of detailed chemistry and was not possible in this PhD.

One of the initial ideas for the work in this thesis was to consider the effect of different glycosidic linkages on the fermentation profile of the molecules. This was perhaps a naïve aim as the existence of these bonds could not be firmly established and certainly, the relative proportions of each linkage in the polymer were impossible to determine.

To establish the physiological impact of the change in SCFA profile caused by pyrodextrin fermentation, human *in vivo* studies were planned to investigate their effect on plasma lipids, faecal SCFA and bacterial populations. The first task to achieve before these studies could be carried out was to obtain a substantial supply of pyrodextrins to allow long-term feeding studies. This proved more difficult than anticipated as food grade material could not be produced in Glasgow and there was not sufficient time to produce such material elsewhere, for instance, in Venezuela. I therefore searched to find an alternative source commercially produced. I first considered gofios from the Canary Islands. This toasted flour should contain pyrodextrins as it is produced in a fashion, which would be predicted to result in starch pyrodextrinisation. However, when tested for their digestibility using the Englyst method, the resistant carbohydrate component was

too small to make them a useful source of pyrodextrins. However, Canary Islands gofios had an important content of slowly digestible starch, a fact that deserves further investigation since gofios are consumed not only by the Spaniard islanders but also in some Northern African and Latin American countries.

The use of Fibersol-2, a commercial non-digestible pyrodextrin from corn starch, was then decided upon although the molecular weight profile of this product was less than 2000, in contrast to the material used in our *in vitro* study (MW 2000 – 105,000), where the smaller molecules were lost in a dialysis process. The fermentation profile of Fibersol-2 however, was similar to that of the higher molecular weight pyrodextrins but a lower proportion of butyrate was produced and slightly more acetate. Most of the work on Fibersol-2 is published in Japanese and although the translated summaries suggest that this pyrodextrin source may reduce plasma lipids, the data is not readily available for scrutiny by most scientists and clinicians so it was considered useful to study its effects in this PhD.

Another major set back in the human trial was the problematic recruitment of hyperlipidaemic men. We had been advised that recruiting men with higher BMI (more than 27 kg/m²) and high waist circumference (more than 91 cm) would result in a high identification of hyperlipidaemia. This did not occur in practise. Although we screened many subjects, only three were identified as hyperlipidaemic and so we were unable to carry out this type of study within the last year of the PhD. In compensation, we decided to concentrate on the colonic effects of Fibersol-2 and so recruited 12 healthy subjects who ate 30 g/d of Fibersol-2 for one week in a crossover design. Faeces were collected and analysed for SCFA and bacteria. In addition, subjects kept a faecal frequency and stool

consistency dairy. This study aimed to test the feasibility of a larger study in patients in terms of the dose of Fibersol-2, the vehicle of Fibersol-2 (yoghurt) and to see if the Fibersol-2 had any prebiotic actions. The use of faeces to test for changes in SCFA profile is not ideal as most SCFA are produced in the proximal colon and may be absorbed before faeces are produced. Indeed, we did not detect an effect of Fibersol-2 on the faecal SCFA in this study. However, in the time available this was the only possibility to get an idea of the actions of the Fibersol-2 on colonic function. In the case of the prebiotic action, although we saw an increased number of lactic acid bacteria in the stool during the Fibersol-2 intake, the effect was small. Indeed, a parallel *in vitro* study of Fibersol-2 fermentation gave no indication of a prebiotic effect. However, the study design was accepted by the subjects and the yoghurt vehicle was successful and well tolerated over a significant period. This suggests it would be suitable for a long-term clinical study. The Fibersol-2 dose (30 g/day) had no major side effects. One subject complained of diarrhoea for 3 days, but an increase in stool frequency was not reported by most subjects.

In conclusion, in this thesis I have established that pyrodextrinisation of starch from lentil, cocoyam and potato changes the structure of the molecule producing low molecular weight species containing anhydro-glucose and larger polymers that are probably highly branched with β glucosidic linkages.

These polymers are non digestible in the small intestine, but highly fermented giving a higher proportion of propionate but similar amounts of butyrate to their native starches. This may have implications for plasma lipid levels in humans, but sufficient *in vivo* studies were not possible and should be studied in future. The

change in SCFA profile may be due to the β bonds in the pyrodextrins or to their solubility. This is not possible to be established firmly as we do not know their true structure. Further study of the low and high MW portions could help to answer this. However, the NMR analysis required to determine the exact structure of these molecules will be very costly and time consuming.

The *in vivo* actions and potential health benefits of pyrodextrins have still to be established. The lipid lowering effects of Fibersol-2 should be confirmed in further studies that could use the design proposed in this thesis but would be best carried out in hyperlipidaemic men. It is not clear if these effects will be related to the higher propionate production. Better methods are needed to determine SCFA production *in vivo* and particularly the amounts of propionate reaching the liver. This is not possible using present techniques but requires flux experiments using stable isotopes or other labelled SCFA. Animal studies with radio labelled SCFA could be used but rodents are not good models for human plasma lipids and cholesterol.

Although pyrodextrins are derived from starch and could be considered a separate category of resistant starch, they may be more similar in structure and function to non-digestible oligosaccharides or other β linked glucans, such as cellulose or oat gum. Indeed, all these non-digestible carbohydrates could now be included in the category of dietary fibre, if the new proposed AACC definition (DeVries, 2003):

“Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes

polysaccharides, oligosaccharides, lignin, and associated plants substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation”

is accepted.

It has been a long journey for starch pyrodextrins since that fire in Ireland where the British gums were accidentally discovered. A journey from an adhesive compound that was used to glue stamps to a food ingredient that may help to reduce the risk of cardiovascular disease. Only the future holds a full understanding of the properties of pyrodextrins and their possibilities. This PhD was only a step to get close to that future.

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Appendices



Notes & Tips

A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates

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In 1955, Roe [1] described a modification of a method to measure dextran in blood and urine [2] for estimation of sugar in blood and spinal fluid. In these methods, total carbohydrates are measured using the anthrone-sulfuric acid reaction. Here, heat and a strong acidic environment produce both hydrolysis of glycosidic bonds and dehydration of monomers to produce furfuraldehyde derivatives. These compounds react with anthrone and produce colored products. However, routine measurement of a large number of samples requires the use of a significant amount of concentrated sulfuric acid and needs strict safety precautions as well as many test tubes. Previous attempts of automation have been hampered by the exothermic nature of this reaction (G. Palacios, personal communication). The use of microtitration plate technology offers a way to overcome these problems and reduce cost, time, and hazard. Therefore, our aim was to adapt the well-known anthrone-sulfuric acid assay for reliable quantification of glucose-based carbohydrates using 96-well microtitration plates.

This assay requires a total of 12 mL of anthrone-sulfuric acid reagent (2 g/L anthrone solution in concentrated sulfuric acid) and 0.5 mL standard solution (0.4 g/L glucose solution in water) per plate. The anthrone-sulfuric acid reagent must be prepared just before use. Aliquots of 24 mg of anthrone were made in bulk into 30-mL universal plastic containers with leak-proof caps and kept protected from light until used. Just before addition of the reagent to the plate, 12 mL of concentrated sulfuric acid, stored at 4 °C, was added to

one universal container and vortex-mixed thoroughly to prepare the reagent.

The microtitration plates employed were 96-well, flat-bottomed, polystyrene microtest plates (Immulon 4, Catalogue Number 011-010-3855, Dynatech Laboratories Ltd., Billingshurst, UK) because they were able to withstand the severe incubation conditions of this assay. In addition, the wells of these plates are joined together at their rim instead of at the bottom. This design allowed each well to be surrounded by water during water bath incubations, promoting a faster and equal distribution of the heat throughout the plate when compared with other plates (Costar, Catalogue Number 9017, Corning Inc., Corning, NY; Ratiolab, Catalogue Number RB/60.1811, Camlab Ltd., Over, UK).

The procedure was as follows: 40 µL water (blank), standard (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.4 g/L glucose), or sample was added to individual wells of the microtitration plate (Fig. 1). This plate was covered with clingfilm, vortex-mixed gently, and incubated at 4 °C for 15 min. Next, 0.1 mL anthrone solution (freshly prepared) was added to each well using a 12-channel micropipette; the plate was sealed with acetate tape (plate sealer catalogue number M30, Dynatech Laboratories Ltd.), vortex-mixed gently but thoroughly, and incubated at 92 °C in a nonshaking water bath. After 3 min, the plate was transferred to a nonshaking water bath at room temperature for 5 min to stop the reaction and then placed into an oven at 45 °C for 15 min to dry off. The 3-min incubation is critical as this allows optimal development of color without deformation of the plate due to the high temperature in the water bath. In this regard, plates should not be left in the water bath for more than 5 min. Absorbance at 630 nm was read in a plate reader (Dynatech MR5000, Dynatech Laboratories Inc., Chantilly, VA) and total carbohydrate concentration, as glucose or starch (0.9× glucose

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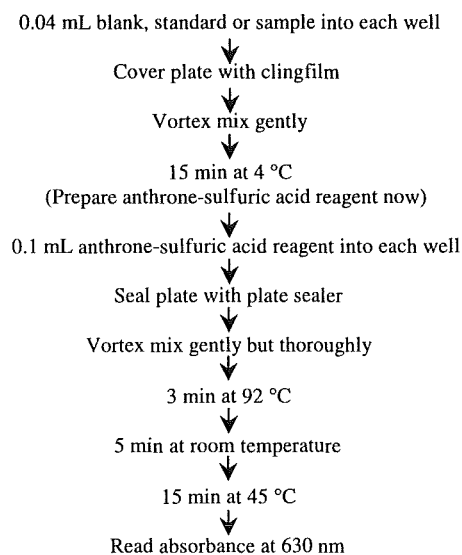


Fig. 1. A flowchart outlining the main steps in the method for analysis of glucose-based carbohydrates by the anthrone-sulfuric acid colorimetric microassay.

concentration) equivalents, was worked out. Under this scheme, it is possible to test 24 samples in parallel with a 7-point standard curve plus blank, by triplicate on each plate. Other combinations may allow even more samples to be tested. The whole procedure was completed within 2.5 h, which represents an important decrease in time, since processing 96 test tubes by the conventional method usually takes 4 h.

A linear curve was obtained within the concentration range used for the standards (0.05–0.4 g/L) represented by the equation: $\hat{Y} = 3.206X + 0.051$, with 0.982 as coefficient of determination (R^2)² and 0.05 as estimated standard deviation (s) about the regression line. Precision was tested using sample solutions (0.2, 0.3, and 0.4 g/L) of either potato pyrodextrin [3] or commercial glucose-based maltodextrin-like oligosaccharides (Fibersol-2, Matsutani Chemical Industries Co. Ltd., Itami, Japan). In both cases, within-run precision was less than 2% ($n = 3$) and between-run precision was less than 4.5% ($n = 5$). In order to test the capacity of these conditions to hydrolyze a high molecular weight polymer, commercial soluble starch (Catalogue Number 33615, Reidel-de Hagaën, Gillingham, UK) was used as standard instead of glucose. The equation obtained ($\hat{Y} = 3.249X + 0.069$, $R^2 = 0.991$ and $s = 0.04$) was similar to that of glucose, suggesting that the polymer was completely hydrolyzed.

There are other methods to estimate total carbohydrate currently in use, especially the method described

by Dubois et al. in 1956 [4], which uses a phenol-sulfuric acid reagent. The Dubois method provides a better way to assay polysaccharides that contain mixtures of different sugar moieties than the assays developed by Roe [1,2]. Anthrone reacts differently with different carbohydrate moieties. As a result it is not accurate to use any one sugar as a suitable standard, when more than one simple sugar is present in the polysaccharide structure. The Dubois method overcomes this limitation by using phenol [4], so a wider spectrum of polysaccharides can be analyzed. However, phenol is carcinogenic and may cause adverse mutagenic and teratogenic effects, whereas anthrone in the Roe method has neither carcinogenic nor other known adverse effects. Therefore, when glucose-based carbohydrates like dextrans, starches, or modified starches need to be analyzed, the anthrone-sulfuric acid assay is the preferred method. It is even more attractive when the assay is performed with small amount of reagents, like the microassay presented here, since this reduces the hazard in manipulating corrosive agents like sulfuric acid.

This novel microassay is very suitable for the study of modified starches. Quantitative analysis of modified starches like pyrodextrans can be underestimated by using highly specific enzymatic assays [5]. This microassay can be used instead, overcoming more complicated and expensive assays like those based on gas liquid chromatography [6]. It can also be used as part of the determination of the depolymerization degree expected from some starch modification procedures by monitoring size-exclusion chromatography without using detectors based on diffractometry [3,7]. This microassay might also be useful in other fields where the anthrone-sulfuric acid assay is being used, such as analysis of carbohydrate mobilization in mosquitoes [8], quantification of soil carbohydrates [9], and characterization of organic matter in domestic wastewater [10].

In summary, the anthrone-sulfuric acid colorimetric microassay developed is suitable for routine analysis of glucose-based carbohydrates. It reduces both assay time (40%) and amount of reagent (8-fold), decreasing the cost and the hazards of the assay. This approach to miniaturize assays that have hazardous chemicals could be also employed for other procedures.

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² Abbreviations used: R^2 , coefficient of determination; s , estimated standard deviation about the regression line.

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ORIGINAL CONTRIBUTION

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Differential fermentation of glucose-based carbohydrates *in vitro* by human faecal bacteria

A study of pyrodextrinised starches from different sources*

Summary Background Pyrodextrins, modified starches produced by heat/acid treatment, have been used extensively in the paper industry. Recently, pyrodextrinisation has been recognised as a way of producing a “resistant starch” that is water-soluble and has non-starch link-

ages. However, a full characterisation of the fermentation properties of pyrodextrins has not been reported. **Aim of the study** To evaluate the effect of pyrodextrinisation on the fermentation characteristics of starches, prepared from Venezuelan crops, in a simple *in vitro* model of the human colon. **Methods** Potato, lentil and cocoyam pyrodextrins were produced using heat (140 °C for 3 h) and hydrochloric acid as catalyst (1.82 g/kg starch). Then, both native and modified starches were pre-digested with pepsin and pancreatic enzymes and their resistant components fermented anaerobically using human faeces as inocula for 24 h. Short-chain fatty acids (SCFA), pH, residual starch and carbohydrate in the cultures were measured. **Results** More than 69 % of initial carbohydrate disappeared from both pre-digested native and pyro-

converted starch cultures. More than 6.8 and 10.0 mmol net SCFA per gram carbohydrate were produced from pre-digested native and pyrodextrinised starches, respectively. In cultures of predigested pyrodextrins, the molar ratio for propionate doubled, whereas the ratio of acetate decreased by 25 % when compared with pre-digested native starches. The ratio of butyrate did not change. **Conclusions** The mechanism for the change in SCFA profile is unclear, but may be related to solubility and/or presence of non-starch linkages. The presence of these bonds may modify the accessibility/affinity of bacterial enzymes to the modified starch structure.

Key words pyrodextrin – short-chain fatty acids – modified starch – pyroconversion – colonic fermentation

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Introduction

Starch modification techniques have been developed for industrial processing to produce a wide range of potential food ingredients, including pyrodextrinised starches. However, interest in modified starches has been restricted mainly to technological aspects with lit-

tle concern about the possible impact of the modification on the digestibility and fermentability of the product [1, 2].

Pyrodextrins are produced by reactions that take place under the influence of heat, often in the presence of catalytic amounts of ions. These modified starches were first reported nearly 200 years ago, as a water soluble and gummy material from the roasting of starch [3]. Native starch is composed of glucose units linked by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glycosidic bonds, which can be broken down by the digestive enzymes present in the small intestine. Starch pyrodextrinisation occurs because of

* Part of this work has been presented in abstract form (Laurentin A, Edwards CA (2001) *In vitro* fermentation of pyrodextrinized lentil and cocoyam starches. Proc Nutr Soc 60:192A).

hydrolysis, transglucosidation and, in some cases, re-polymerisation reactions of glucans. After hydrolysis, the new reducing end group of the glucose chain becomes a glucosyl cation, which can undergo either inter-molecular bond formation between two chains or intramolecular dehydration, leading to the formation of 1,6-anhydro- β -D-glucose [4, 5]. Therefore, pyroconverted starches are branched, low molecular weight products showing new glycosidic linkages. It is claimed in the European patent EP 0 540 421 A1 that the non-digestible fraction of a potato pyrodextrin had one-third of its glucose residues at the non-reducing end of a chain, and one-sixth of glucose residues presented atypical linkages, such as (1 \rightarrow 3) and (1 \rightarrow 2). In addition, four main groups of molecular size for pyrodextrinised maize starch with apparent Mw at 5,000; 10,000; 19,000 and 40,000 have been described [6].

Although it has been recognised for some time that pyrodextrins prepared without any catalyst added were resistant to digestion [7], data about the digestibility and fermentability of pyrodextrins (with or without catalyst) are not readily available. Recently, other authors have found not only that acid catalysed pyroconversion promoted the generation of a significant proportion of non-digestible fractions (around 60%), but also these changes differed in magnitude depending on the starch source [2, 6]. A decrease in rat caecal content pH and an increase in butyrate was reported when rats were fed with corn pyrodextrin enriched diet in comparison with a corn fibre diet [8]. However, full characterisation of the fermentation of pyrodextrins from other plant sources and in comparison to their native starches has not been reported.

In recent years, resistant starches have been recognised for the contribution they can make to human health throughout their interaction with the gut. In particular, the increased butyrate production as this short-chain fatty acid (SCFA) may have anticancer and anti-inflammatory effects on the colo-rectal mucosa [9, 10]. Pyrodextrinised starches share some of the properties of resistant starch. They are partially fermented, producing more butyrate than non-starch polysaccharides and a low colonic pH [8]. On the other hand, pyrodextrinised starch, but not resistant starch [11], decreased gut transit time in rats [12] and may lower serum cholesterol and neutral fat in humans [13]. As pyrodextrinised starches have different glycosidic bonds from resistant starch, they may be fermented by different bacterial species and both the process and end result of fermentation may differ.

The aim of this work, therefore, was to evaluate the effect of pyrodextrinisation on the fermentation characteristics of starches, prepared from potato, lentil and cocoyam, as a substrate in a simple *in vitro* model of the human colon.

Materials and methods

Starch sources

Lentil (*Lens esculenta* Medic.) seeds and cocoyam (*Xanthosoma sagittifolium* (L.) Schott.) roots were purchased from a local market in Caracas (Venezuela). Commercially isolated potato starch was purchased from Lyckeby Stärkelsen (Kristianstad, Sweden).

Starch isolation

Starch was isolated as described elsewhere [14]. Briefly, decorticated lentil grains or peeled and diced cocoyam roots were homogenised in a liquidiser using one volume of water. The suspension was filtered through a 280-mesh cloth several times (usually 7 times), each time adding one volume of water, until no material passed through the cloth. The filtered material (essentially starch) was washed out with an equal volume of water, three or four times by centrifugation (1,500 g for 15 min), removing the lipid layer out of the surface in each centrifugation step until none appeared. Before the last washing, the pH was adjusted to neutrality. Then, the starch was dried in an oven at 45°C for 24 h, sieved out (250 μ m pore), and stored at room temperature. Additionally, lentil grains were steeped in 62.5 mmol/l NaOH solution for 24 h at room temperature and washed out extensively afterwards, as a pretreatment to facilitate the peeling of the grains.

Yield was 24% and 45% (d. b.) for lentil and cocoyam starches, respectively. It was low as the method used [14] was developed for the isolation of amaranth starch and no attempt to improve the yield for other sources was made because the emphasis was put on a high starch purity. The estimated starch content of the preparations used in this study was 94.7% and 96.8% for lentil and cocoyam, respectively [6]. Additionally, the protein content was 0.24% for lentil [15] and 0.56% for cocoyam [16] starches. Therefore, the starting materials for the pyrodextrinisation step were mainly starch and the impact of other components, such as antinutritional factors, was most likely minor.

Starch pyrodextrinisation

Isolated starches (22 g) were sprayed with 0.5 ml HCl (1.82 g acid/kg starch), mixed thoroughly and left overnight at room temperature. Then, they were heated at 140°C for 3 h, milled and sieved through a 250 μ m pore size mesh [17].

Starch pre-digestion

To remove the digestible starch, both native and pyrodextrinised starches were pre-digested using an Englyst starch kit (Englyst Carbohydrate Services Ltd, Cambridge, UK) with some protocol modifications. Briefly, 0.8 g starch and 50 mg guar gum were weighed in a centrifuge tube, then 10 ml of 5 g/l pepsin suspension in 50 mmol/l HCl were added, vortex mixed and incubated for 30 min at 37 °C. To simulate small intestinal digestion, five glass balls and 10 ml 0.25 mol/l sodium acetate solution were added to each tube and they were shaken well by hand. After equilibrating at 37 °C, an enzyme mixture (pancreatin from porcine pancreas, amyloglucosidase from *Aspergillus niger* and invertase from yeast; 5 ml) was added and incubated for 2 h in a shaking water bath (70 strokes/min). Then, the glass balls were taken out and the tubes were spun at 1,500 g for 5 min. The non-digestible fraction from the pre-digested native starches was recovered from the pellets, freeze-dried, pooled, milled in a mortar, and used for the fermentation experiments. This fraction contained the resistant starch, as defined by Englyst et al. [18], and guar gum.

In contrast, the non-digestible fraction from the pre-digested pyrodextrinised starches remained in the supernatant after centrifugation, due to their solubility in water. However, as the products of the pre-digestion step were also present in the supernatant, these were removed by dialysis (overnight dialysis against water at 4 °C, followed by five 1-hour repeat dialysis steps with stirring) using dialysis bags with small Mw cut off (2,000). Retentates were pooled, freeze-dried, milled in a mortar, and used for fermentation. This fraction contained the non-digestible component from the pre-digested pyrodextrin and the enzymes from the starch kit.

As the SCFA profile was one of the variables studied, it was important to consider the effect of other fermentable substrates present in the system. Therefore, potato native and pyroconverted starches were fermented as described below, but without the pre-digestion step to look at the impact of the presence of either guar gum or the enzymes (from the starch kit) in the non-digestible fractions obtained from native or pyrodextrinised starches, respectively. There were no discernible effects of either component.

Fermentation of non-digestible fractions

Non-digestible fractions (and glucose, as a readily fermented control) were fermented according to Edwards et al. [19] in four to six independent determinations (actual sample size is given in Table 1). Faecal slurry was made using freshly voided faeces (within 45 min from evacuation), donated by healthy adults (34–36 years, one female), homogenised in 0.1 mol/l Na,K-phosphate

buffer, pH 6.5 (pre-boiled, cooled and kept in an oxygen free nitrogen (OFN) atmosphere until used) using a liquidiser. Starch samples (100 mg for test cultures or none in case of control cultures) were suspended in 10 ml 160 g/l faecal slurry (previously filtered through a nylon stocking) in McCartney bottles (28 ml capacity). Each bottle was fitted with a holed, screw cap with a rubber lining to allow flushing of the culture with OFN before incubation. The bottles were incubated horizontally in a shaking water bath (50 strokes/min) at 37 °C for 24 h.

After incubation, produced gas was released and measured using a calibrated syringe. A culture aliquot (2 ml) was used to measure pH and frozen at –20 °C for later SCFA analysis. The remaining slurry was boiled for 30 min and frozen to assess residual starch and carbohydrate. Time zero cultures were immediately boiled for 30 min and frozen to estimate total starch and carbohydrate.

Short-chain fatty acid assay

SCFA were estimated by gas liquid chromatography using a TRACE™ 2000 gas chromatograph (ThermoQuest Ltd, Manchester, UK) equipped with a flame ionisation detector (250 °C) and using a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 µm film thickness), made of polyethylene glycol (catalogue No. 7EK-G007-22, Phenomenex, Cheshire, UK). Nitrogen (30 ml/min) was used as the carrier gas. Internal standard solution (86.1 mmol/l 3-methyl-n-valeric acid, 0.1 ml) and concentrated orthophosphoric acid (0.1 ml) were added to 0.8 ml culture aliquots. The mixture was extracted three times with 3 ml diethyl ether each time, centrifuged and the ether layers pooled. One microlitre of ether extract was automatically injected (230 °C, splitless) into the column. Then, the column temperature was held at 80 °C for 1 min, increased at 15 °C/min until 210 °C and held for 1 min. The peak integrals were analysed using Chrom-Card 32-bit software version 1.07β5 (2000) by ThermoQuest (Milan, Italy) using an averaged (n=5) response factor for each external standard (166.5 mmol/l acetic, 135.0 mmol/l propionic, 113.5 mmol/l isobutyric, 113.5 mmol/l n-butyric, 97.9 mmol/l isovaleric, 97.9 mmol/l n-valeric, 86.1 mmol/l n-hexanoic, 76.8 mmol/l heptanoic and 69.3 mmol/l n-octanoic acid solutions, pH 8) as calibration method. All the standards were from Sigma-Aldrich Company Ltd. (Dorset, UK), except acetic acid glacial, which was from Fisher Scientific (Loughborough, UK).

Starch and carbohydrate analyses

Starch and carbohydrate were estimated both before (total) and after (residual) 24 h anaerobic incubation to

quantify the degree of fermentation. Total starch was measured using the enzymatic procedure described by Englyst et al. [18]. Residual starch was estimated according to Edwards et al. [19] based upon Englyst et al. [18]. These two methods are essentially the same and quantify the starch content as glucose released after an *in vitro* simulation of both gastric and small intestine enzymatic digestion. The specificity of the Englyst method, however, underestimates the "starch" content in modified starches such as pyrodextrins because of the presence of atypical bonds that cannot be hydrolysed by the enzymes used in the method. Therefore, measurement of carbohydrate by a more general, chemical assay was necessary to estimate the degree of fermentation of the pre-digested pyrodextrins.

Total and residual carbohydrates were estimated, by sampling from the same preparations made for total and residual starch analyses, using the anthrone-sulphuric acid method, which is a suitable method for the estimation of glucose-based carbohydrates like starches [20, 21]. A modification was made to perform the assay into 96-well microtitration plates. The reaction was carried out mixing 40 µl sample, standard or blank with 100 µl 10.3 mmol/l anthrone in concentrated sulphuric acid and incubated at 92 °C for 3 min. It has been shown that these assay conditions are able to quantify high molecular weight polymers like commercial soluble starch [21]. Absorbance at 630 nm was read using a Dynatech MR5000 microplate reader and analysed with Dynatech Reader software version 1.1 (Dynatech Laboratories Inc., Chantilly, VA, USA). An appropriate calibration curve was made with each plate using glucose as standard [21]. Standard solutions for both starch and carbohydrate determinations were prepared dissolving glucose in slurry supernatants (1,500 g for 5 min) from control cultures at time zero.

Statistical analysis

Variables were described as mean \pm standard deviation. Statistical analysis was made using Minitab® for Windows software, release 10.51 Xtra (Minitab Inc., State College, PA, USA). One- or two-sided unpaired t-test was used to compare means of pre-digested pyrodextrinised against pre-digested native starches where appropriate. A probability level less than 0.05 was used to indicate a significant difference between means.

Results

Fermentation of native and pyrodextrinised starches

The fermentation properties of both pre-digested native and pyrodextrinised starches from several sources after

24 h *in vitro* anaerobic incubation with human faeces are shown in Table 1. In cultures containing the pre-digested native starches, 99, 98, and 95 % of potato, lentil, and cocoyam starches, respectively, were fermented. Although a similar trend was found for the pre-digested pyrodextrinised samples, the modified starch was measured as carbohydrate content by a chemical method [21] to overcome the specificity of the enzymatic starch assay [18]. With this approach, 77, 75, and 81 % of the carbohydrate in potato, lentil, and cocoyam pre-digested pyrodextrins were fermented, respectively.

Total SCFA

The net total SCFA, estimated as mmol/l of fermented culture, was similar for all starches (Table 1). Comparable amounts were produced using glucose as substrate (58.8 ± 4.4 mmol/l, $n = 6$). However, when net total SCFA were corrected for initial culture carbohydrate content, SCFA production remained similar for all the pre-digested native starch sources, but the total SCFA for the pre-digested pyrodextrinised starches were significantly higher (between 43 % and 75 %) than their corresponding predigested native starches ($p < 0.023$, one-sided t-test). Control cultures ($n = 6$), i. e. those fermented without starch samples, produced 32.4 ± 9.1 mmol/l of total SCFA, achieved pH 6.5 ± 0.1 and produced 7 ± 3 ml gas.

Molar proportions of SCFA

All pre-digested pyrodextrins showed a significantly higher ($p < 0.017$, one-sided t-test) molar ratio of propionate (around twofold) when fermented compared with their pre-digested native starch. In addition, the acetate molar ratio decreased ($p < 0.04$, one-sided t-test) by about 24 % (Table 1). There was no difference in the n-butyrate molar proportion ($p > 0.82$).

Fall in pH and gas produced

The culture pH was 6.6 ± 0.1 ($n = 6$) at time zero. After fermentation, all the cultures showed a decrease in pH, but the fall in pH was significantly less for the pre-digested pyroconverted samples ($p < 0.03$, one-sided t-test). The fall in pH observed for glucose (1.7 ± 0.3 , $n = 6$) was similar to all the pre-digested native starches (Table 1). The volume of gas produced was the same for all samples, including glucose (17 ± 5 ml, $n = 6$).

Table 1 Short-chain fatty acid, fall in pH, gas produced, starch and carbohydrate contents in fermented cultures of pre-digested native and pyrodextrinised starches from potato, lentil and cocoyam^b

	Starch source (pre-digested starches)					
	Potato		Lentil		Cocoyam	
	Native (n = 5)	Pyrodextrin (n = 4)	Native (n = 6)	Pyrodextrin (n = 6)	Native (n = 4)	Pyrodextrin (n = 6)
Net total SCFA ^c (mmol/l)	55.4 ± 5.8	61.0 ± 5.8	58.2 ± 5.0	56.6 ± 4.4	60.1 ± 14.5	59.1 ± 5.4
Net total SCFA ^{a, c} (mol/kg carbohydrate)	6.9 ± 0.7	10.4 ± 1.9*	7.0 ± 0.4	10.0 ± 0.7***	6.8 ± 2.3	11.9 ± 1.8*
Individual SCFA molar ratio						
Acetate ^a	649 ± 126	511 ± 35*	688 ± 121	514 ± 30**	660 ± 70	513 ± 31*
Propionate ^a	147 ± 34	254 ± 59*	120 ± 32	243 ± 56**	111 ± 17	244 ± 52***
n-Butyrate	154 ± 83	151 ± 51	148 ± 79	156 ± 41	161 ± 29	161 ± 41
iC4–C8 ^a	50 ± 32	85 ± 41	44 ± 34	87 ± 32*	69 ± 48	82 ± 37
Fall in pH ^a from 6.6 ± 0.1	1.5 ± 0.3	1.0 ± 0.1**	1.6 ± 0.3	0.9 ± 0.1***	1.4 ± 0.2	1.0 ± 0.1*
Gas produced (ml)	16 ± 3	20 ± 4	14 ± 8	17 ± 3	10 ± 5	15 ± 6
Total starch ^{a, d} (mg SE)	78.8 ± 2.4	5.6 ± 1.8***	85.7 ± 11.0	7.0 ± 6.5***	90.5 ± 3.2	5.6 ± 3.3***
Residual starch ^{a, e} (mg SE)	1.1 ± 0.7	0.8 ± 0.9	1.6 ± 1.1	0.7 ± 0.6	4.3 ± 2.4	0.6 ± 0.6*
Total carbohydrate ^{a, d} (mg SE, n = 4)	77.7 ± 8.1	59.8 ± 8.3*	80.5 ± 2.3	55.7 ± 3.9***	87.3 ± 2.9	50.2 ± 4.3***
Residual carbohydrate ^{a, e} (mg SE, n = 4)	7.4 ± 6.0	13.5 ± 4.4	4.1 ± 3.6	13.9 ± 2.2*	27.1 ± 13.3	9.6 ± 2.2

Values are means ± standard deviations

SCFA, short-chain fatty acids; iC4–C8, sum of iso-butyrate, iso-valerate, valerate, hexanoate, heptanoate and octanoate; SE, starch equivalents (0.9 x glucose concentration)

Mean values were significantly different from their corresponding predigested native starch: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (unpaired t-test, one-sided)

^b Fermentation was carried out in anaerobic incubations with human faeces for 24 h

^c Net total SCFA was calculated by subtracting total SCFA values in control cultures (no carbohydrate) from total SCFA values in test cultures. Total SCFA in faecal slurry (at time zero) was 11.4 ± 4.8 mmol/l (n = 6)

^d Before fermentation

^e After fermentation

Discussion

Maize, potato and cassava starches account for almost 90% of the starch produced in the world [22]. To widen the utilisation of underexploited crops such as lentil and cocoyam, produced in Latin American countries like Venezuela, as starch sources, it is important to study their characteristics as potential food ingredients. Previous work has shown that pyrodextrinised starches produced by the acid/heat treatment used here have a 55–65% decrease in the enzymatically available starch. Therefore, pyrodextrins are an excellent source of non-digestible carbohydrates [6]. Moreover, these pyrodextrins differ from other short-chain saccharides because they are composed of a complex mixture of starch derivatives, all with Mw below 105,000 as estimated by gel filtration chromatography [6].

However, the water solubility of the pyrodextrins caused an analytical problem. During the pre-digestion stage, the nondigestible fraction from pre-digested native starches is normally obtained as a pellet after centrifugation at the end of pre-digestion [18]. In contrast, the pyrodextrinised starches remain in the supernatant along with their products of digestion (mainly glucose) and with the enzymes used for the pre-digestion itself. A

dialysis step with small Mw cut-off (2,000) was used to remove the digestion products before fermentation. However, low Mw pyrodextrins, which might be non-digestible and fermentable, were also lost during this step. Consequently, pyrodextrins used in this work had Mw in the range of 2,000 to 105,000.

Pyrodextrins do not belong to any of the three categories of resistant starch originally proposed [18]. Some authors have suggested new categories of resistant starch to include not only chemically modified starches [23], but also physically modified starches (like extruded starch) and amylo-lipid complex [11]. Since pyrodextrins have different glycosidic bonds than those present in native starches, they may belong to the category that includes chemically modified starches.

There was an almost complete fermentation of all starch samples under the fermentation conditions used [19] based on net total SCFA production, fall in pH, starch and carbohydrate content before and after 24 h anaerobic incubation (Table 1). Net total SCFA and pH for pre-digested native starches were very similar to those reported elsewhere for raw potato starch [19]. Although raw and native starches are not part of the western diet, they were used here for comparison purposes. The heat/acid treatment did not change the production of gases in the faecal cultures.

Two approaches were used to assess the amount of substrate consumed during fermentation. An enzymatic, highly specific approach using Englyst method, as described by Edwards et al. [19], was used for the pre-digested native starches. On the other hand, a more general, chemical approach using a modification of the anthrone-sulphuric acid assay [21] was used for the pre-digested pyrodextrins. These methods differ in their specificity and they were used because the Englyst method underestimated the starch content of pyrodextrins, as shown under 'Total starch' in Table 1. This was possibly due to the presence of non-starch linkages (i. e. not $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow6)$ bonds) in such modified starches, which cannot be hydrolysed by the enzymes used in the assay.

Regarding the SCFA profile, pre-digested pyrodextrin fermentation showed the high proportion of butyrate that characterises native starch fermentation [24], yet there was an increase in the molar ratio of propionate with a parallel decrease in acetate. Another study [25] has shown a lower propionate ratio in raw potato and banana starches (resistant starch type 2) when compared with both wheat starch (digestible starch) and retrograded wheat and maize starches (resistant starch type 3). However, differences seen in the present study were 2- to 6.5-fold larger than those previously reported [25]. The drop in pH was higher for the pre-digested native starches than for pyrodextrins (Table 1). This may be due to the higher proportion of acetate, which has a lower pKa (4.74) than the other SCFA, present in the native sample cultures.

It is interesting to note that the only carbohydrate moiety present in pyrodextrins is glucose. The changes during pyrodextrinisation of native starches yield oligo- or polysaccharides with lower Mw than native starches,

along with the new glycosidic bonds. The extent of these changes depends on the condition employed [4], but usually render highly branched [13], water soluble, resistant-to-digest pyrodextrins [6]. It is not clear why pre-digested pyrodextrins had a higher propionate production than their pre-digested native starches. However, it could be due to an increased solubility and/or presence of non-starch bonds caused by transglucosidation reactions. The presence of these new, atypical bonds may change the starch structure in a way that modifies the accessibility and/or affinity of the bacterial enzymes to the bonds.

High solubility, on the other hand, may also be important. α -Glucooligosaccharide (branched pentasaccharide with 1 \rightarrow 2, 1 \rightarrow 4 and 1 \rightarrow 6 α -bonds) and maltodextrin-like oligosaccharides (branched oligosaccharides with 2,000 Mw and 1 \rightarrow 4, 1 \rightarrow 6, 1 \rightarrow 2 and 1 \rightarrow 3 α - and β -bonds), both highly soluble and resistant-to-digest carbohydrates yielded similar SCFA molar ratio to the pre-digested pyrodextrins (Table 2) when fermented with human faecal microflora *in vitro* [26].

However, insoluble β -bonded fibres also show a SCFA profile similar to the pre-digested pyrodextrins. A molar ratio of 653:257:90 for acetate, propionate and butyrate, respectively, was reported when oat husk (44% cellulose, 50% hemicellulose + pectin) was used as fibre source in a diet for rats inoculated with human faecal flora [27]. Oat bran (mainly β -glucan) [28] and cellulose [29] fermentation *in vitro* also yielded a high propionate molar ratio (Table 2). Acid catalysed pyrodextrins, as prepared in this study, are thought to contain a significant proportion of β -bonded glycosidic linkages, as shown for wheat starch heated during 4 h at 180 °C without addition of any catalyst [7]. However, the presence of such β -bonds in acid catalysed pyrodextrins has yet to be confirmed.

Table 2 Short-chain fatty acid molar ratios for several glucose-based carbohydrates in *in vitro* cultures of human faeces or in human faecal associated rats *in vivo*

Carbohydrate	SCFA molar ratio*			Soluble in water	Glycosidic bond ^a	Reference
	Ac	Pr	Bu			
Glucose	640	224	136	yes	none	This work
α -Glucooligosaccharide	598	239	164	yes	α	[26]
Maltodextrin-like oligosaccharide	619	301	80	yes	α and β	[26]
Pre-digested pyrodextrin ^b	560	270	170	yes	α and β	This work
Pre-digested native starch ^b	704	133	163	no	α	This work
Cellulose ^c	409	519	72	no	β	[29]
Oat husk ^d	653	257	90	no	β	[27]
Oat bran ^e	560	220	220	no	β	[28]

SCFA short-chain fatty acids; Ac acetate; Pr propionate; Bu butyrate

* Mean values in molar ratio based on net total SCFA (Ac + Pr + Bu)

^a Conformation of glycosidic bonds

^b Mean data from the present study

^c From Sigma (St. Louis, MO, USA)

^d Mainly cellulose and hemicellulose + pectin; in human faecal flora associated rats

^e Mainly β -glucan

In conclusion, pyrodextrinisation of starches isolated from potato, lentil and cocoyam resulted in the production of a soluble material that escapes digestion, but was extensively fermented in vitro by the colonic bacteria of healthy adults. This fermentation is characterised by higher proportions of propionate and lower proportions of acetate than the native starch, although the high pro-

portion of butyrate characteristic of starch fermentation was maintained.

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Full project title as per protocol¹

Effect of modified maize starch on fasting and postprandial serum lipids in healthy adults

Researchers

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Principal research question

Does modified maize starch (Fibersol-2) intake reduce fasting and postprandial lipids in plasma by changing the production of short-chain fatty acids (increasing propionate/acetate ratio) from colonic fermentation in healthy adults?

Duration of project

Six months.

In lay terms, briefly summarise the protocol and state any potentially traumatic procedures and foreseen risks to patients.

Subjects: Thirty healthy men and women (BMI less than 27 kg/m²) between 30 and 65 years old will be studied. Subjects will be excluded if they are taking any medication, being on a special diet, involved in planned exercise, pregnant or planning to become pregnant during the study period.

Fibersol-2: Fibersol-2 is a commercial modified starch, generally known as pyrodextrins, produced from maize starch. Pyrodextrins are produced by heating starches, usually in the presence of an inorganic acid as a catalyst. Fibersol-2 is soluble in water and escapes digestion from the small intestine. The solubility of this modified starch means that, unlike dietary fibre, it can be added to drinks and foods without affecting their properties and therefore is easy to incorporate in the diet of individuals resistant to change their habits. Fibersol-2 will be supplied (free of cost) by Matsutani Chemical Industries Co. Ltd. (Itami, Japan), stored and prepared under food grade conditions.

Experimental design:

Subjects will be randomly divided into test and control groups. Subjects in test group will be asked to consume, during breakfast, 30 g of Fibersol-2 per day mixed with natural yoghurt for 6 weeks (between days 8 and 50). Subjects in control group will be asked to consume just natural yoghurt during the same period. Fibersol-2 plus yoghurt and yoghurt alone will be supplied to the subjects. Oral fat tolerance test (OFTT) will be conducted before and after 6 weeks of the supplementation (on days 8 and 50). Age, sex, weight and height will be recorded on day 1 and weight only on days 8 and 50. A food intake questionnaire will be administered to all subjects on days 1 and 50. Subjects will be asked to provide

¹ This text was based on the "Ethics – Scientific Background" section of the application form submitted to the Glasgow Royal Infirmary Research Ethics Committee, approved on 10 April 2003 (Reference 3HU004) and amended on 6 June 2003 and 15 August 2003.

faecal and urine samples on days 8 and 50. A week before the first OFTT (on day 1), approximately 20 mL of blood (amount equivalent to four teaspoons) will be taken after an overnight fasting (i.e. 12 hours without eating) to measure fasting triglycerides, total-, and HDL-cholesterol. Subjects will be asked to keep to their regular diet and life style throughout the study.

Oral fat tolerance test (OFTT): During each OFTT, a qualified person will introduce a cannula in the forearm for blood sampling. Blood samples (approximately 240 mL, equivalent to half the amount used for donating blood) will be taken in the fasting state (12 hours without eating) and at 15, 30, 45, 60, 75, 90, 120, 180, 240, 300, and 360 minutes (6 hours) after the completion of a high fat meal (test meal). For two days leading up to the first OFTT, subjects will keep a diary of both food intake and physical activity. Subjects will be instructed to replicate their food intake and physical activity before the next OFTT. Blood samples will be analysed for total-, VLDL-, and chylomicron-triglycerides, total- and HDL-cholesterol, insulin, glucose, and non-esterified fatty acids (NEFA) in the Department of Pathological Biochemistry, Royal Infirmary.

Test meal: The test meal for the OFTT will be cereal, coconut, nuts, chocolate, fruit and whipping cream. It will supply according to the subject's body mass (per 1 kg body mass: 1.2 g fat, 1.1 g carbohydrate, and 0.2 g protein; it will provide 1120 kcal for 70 g person).

Faecal sample: For those subjects willing, a fresh faecal sample will be brought to the laboratory within 1 hour after defecation on days 8 and 50. Alternatively, research subjects will produce the faecal sample on the premises. Faecal samples will be analysed for pH, water content, short-chain fatty acid content (by gas chromatography) and residual carbohydrate (by colorimetric assay).

Urine sample: Subjects will be asked to collect urine for 24 hours during the day that OFTT is carried out (on days 8 and 50). Urine sample will be analysed for short-chain fatty acid content using gas chromatography-mass spectrometry.

Potentially traumatic procedures to patients

The only potentially traumatic procedure in this study is blood sampling from an arm vein. Venous cannulation incurs a risk of minor bruising. There is also a risk of thrombophlebitis at the site of cannulation, but this risk is small in subjects with no history of coagulation disorders. Plastic or air embolism can occur, if incorrect cannulation technique is employed. All blood samples will be taken by trained and experienced personnel to minimise the risk to the subjects.

Pyrodextrins, including Fibersol-2, are considered as generally recognised as safe (GRAS) food. Fibersol-2 might give a sandy aftertaste for a few minutes after ingestion. In some subjects, flatulence might be observed, but this will be small and should reduce after a few days.

No other side effects are expected, but there will be medical supervision.

Summary of Aim and Background (200-250 words max.)

The incidence of heart disease has increased over the last few decades and is the highest cause of death. In the West of Scotland, we have one of the highest rates in the world. Increased fasting and postprandial concentrations of triglycerides have been recognised as independent risk factors for coronary heart disease. There is also evidence to suggest that diet can influence triglyceride metabolism.

Fibersol-2, a pyrodextrin made from maize starch, is resistant to digestion in the human small intestine; therefore, it is fermented by the bacteria present in the human colon. The main products of this fermentation process are short-chain fatty acids (SCFA) as well as gases. Some of these SCFA have been suggested to influence lipid metabolism in liver, decreasing triglycerides synthesis. In fact, studies have shown a decrease in fasting triglycerides after Fibersol-2 intake in humans. In addition, we have already shown that several pyrodextrins (including Fibersol-2) significantly changed SCFA during an in vitro incubation with human faecal bacteria over 24 hours. The purpose of this study is to find out if the production of SCFA changes in a similar way when healthy adults consume Fibersol-2 and whether this is associated with beneficial changes in fasting lipids in plasma.

Methodology

- Randomised controlled trial
- Laboratory study
- Questionnaires / interviews

Description of outcome measures

Biochemical measures

Plasma VLDL–LDL sub-fraction by cumulative density gradient centrifugation (compositional analyses)

Plasma LDL sub-fraction by density gradient ultracentrifugation (concentration)

Plasma HDL₂ and HDL₃ sequential (compositional analyses)

Plasma lipoprotein like remnants (cholesterol and triacylglycerides)

Plasma total-triglycerides

Plasma total-cholesterol

Plasma non-esterified fatty acids (NEFA)

Plasma glucose

Plasma insulin

Plasma CRP

Plasma TNF α

Plasma IL6

Faecal short-chain fatty acids

Faecal residual carbohydrate

Urine short-chain fatty acids

Anthropometrical measures

Height

Body weight

Body mass index

Waist circumference

Food intake and physical activity

Diaries and questionnaires

Number of subjects being recruited locally

N=30 (15 in control group and 15 in test group), men and women

Subject inclusion criteria

Age: 30–65 years

BMI less than 27 kg/m²

Fasting triglyceride levels more than 1.5 mmol/L

Subject exclusion criteria

Pregnancy or planning to get pregnant

On medication or special diet

Involved in planned exercise

Source of sample group

Volunteers from the public to the Division of Developmental Medicine recruit by advertisements

Who has given statistical advice on the size, power and design of the project?

Dr David Young, Statistician, Research and Development, Yorkhill NHS Trust

Give details of the literature search carried out.

A critical review of the literature on pyrodextrins (including Fibersol-2) carried out by Mr. Laurentin revealed no studies assessing the impact of pyrodextrin intake on lipid metabolism in the postprandial state. The proposed study is novel. There are several studies on the effect of Fibersol-2 on lipids in the fasting state. A comprehensive review of these papers was hampered because they were all published in Japanese. However, it can be concluded that intake of 29.4 (range 11.7 – 60.0) g/d of Fibersol-2 for 4 weeks decreased fasting plasma triglycerides by 27 (range 19 - 45) % from their initial levels, 2.32 (range 2.00 – 3.22) mmol/L (n=7).

Please indicate which activity area this project falls within

- Gastroenterology, ENT & Ophthalmology
- Healthcare & Diet (includes Nutrition; Nursing; PAMs; General Practice; Primary Care; Health Economics)

FS Study

Food Intake and Stool Frequency Diaries

FS Study
Human Nutrition at Glasgow
Yorkhill NHS Trust
Glasgow, G3 8SJ

Phone: (0141)201-0502

2003

FS Study

NAME:DATE:

FOOD DIARY

Thank you for agreeing to be a subject for our investigation about the effect of modified maize starch on faecal short-chain fatty acids in healthy humans. We hope that you will find the experience interesting.

We need to ensure that conditions for all tests are as similar as possible. We ask you, therefore, to record your **food intake** during the three days before **baseline day**, and then to use this information to ensure that you do not eat differently during the three days before **end-of-intervention day**.

Guidelines for recording food intake

Describe how was the food prepared and cooked

- Specify grilled, fried, boiled or roasted.

e.g.: *grilled, fried, boiled or roasted* meat, mince, fish, poultry, etc.

- Specify type, number and size of meat, fish, poultry, etc.

e.g.: *1 large or medium steak; 2-3 small pork chops, 2 large sausages, 1/2 of whole chicken breast; 1 small hamburger; 1 small chicken leg or thigh*, etc.

Describe the type, size, number of food that was eaten

- Type: e.g. *white or brown* bread, *fruitcake* etc, *chocolate digestive* biscuits, etc.
- Number, size of slices: e.g.: 2 slices (white bread); 2 large slices (fruitcake); 2 units (biscuits)

Use household measures (spoonfuls, bowls, cups) to estimate the amount of food (vegetables, rice, pasta, etc).

- *Spoonfuls*: specify number of teaspoon (**tsp**), dessertspoon (**dsp**), tablespoon (**tb**) or large serving spoon (**ss**).
- *Bowls*: specify 1/4, 1/3, 1/2, 2/3, 3/4 or 1 bowlful
- *Cups*: specify 1/2 or 1 cupful, etc.
- *Volume*: specify glasses, cups, mugs, bottles, cans, pints, litres or volume in ml.

Record food and drinks **as you have them** rather than wait until the end of the day.

To give you an idea of what sort of information you need to fill in, look at the examples given below.

FOOD INTAKE DIARY

Time	Food-drink description / amount per day eaten
7:30 am	3 Weetabix or 1/2 cup cornflakes, 1/2 pint semi-skimmed milk, mug coffee
10:30 am	Cup coffee, 2 chocolate digestive biscuits
1:00 pm	1 sandwich (2 square slices of white bread, 1 slice of ham, 1 slice of cheese, 2 small slices of tomato), 1 medium apple, 1 can of coke
3:00 pm	1 large slice of chocolate cake
6:00 pm	1 small chicken leg or thigh (roasted); 1 cup of boiled vegetables or 2 large serving spoons fresh salad; 1/2 cup of mashed potatoes, 1/2 pint soft drink
	Etc.

FOODS HIGH IN FIBRE

- Cereals with high fibre content (e.g. All-bran, Shredded wheat, Weetabix)
- Whole-meal bread, Crisp bread (e.g. Riveta)
- Foods high in bran, wheat, oat and wheat bran, oat bran flakes, wheat flour, wheat germ
- Beans and lentils (e.g. baked beans, broad beans, kidney and butter beans)
- Fruit and Vegetables

FOOD INTAKE DIARY

CODE:

DAY 1

DATE:

Time	Food-drink description / amount per day eaten

STOOL FREQUENCY DIARY

CODE:

Please fill in the date and time of **each stool passed** and tick the corresponding box you feel is most appropriate for part **a** and **b**. Use next page for any other comment.

Date	Time	Stool characteristics
		a) <input type="checkbox"/> hard pellets <input type="checkbox"/> solid stool <input type="checkbox"/> soft formed <input type="checkbox"/> porridge like <input type="checkbox"/> liquid b) <input type="checkbox"/> easy to pass <input type="checkbox"/> hard to pass
		a) <input type="checkbox"/> hard pellets <input type="checkbox"/> solid stool <input type="checkbox"/> soft formed <input type="checkbox"/> porridge like <input type="checkbox"/> liquid b) <input type="checkbox"/> easy to pass <input type="checkbox"/> hard to pass
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Any notes or comments...

...like rumble (i.e. occurrence of sound, which is different to the one when you are hungry), flatulence, distension of the abdomen, etc.